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LIST OF CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.

JOHN STANLEY DURRANT BACON, *Carbohydrate Biochemistry Department, The Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland* (1)

GUY G. S. DUTTON, *Department of Chemistry, The University of British Columbia, Vancouver V6T 1W5, British Columbia, Canada* (9)

ALAN D. ELBEIN, *Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284* (227)

GEORGE A. JEFFREY, *Department of Crystallography, University of Pittsburgh, Pittsburgh, Pennsylvania 15260* (445)

DAVID JOHN MANNERS, *Department of Chemistry, Heriot-Watt University, Edinburgh EH1 1HX, Scotland* (1)

J. JOHN MARSHALL, *Department of Biochemistry, University of Miami School of Medicine, Miami, Florida 33152* (257)

RAMON L. SIDEBOTHAM,* *Department of Chemistry, Royal Holloway College, London University, Englefield Green, Surrey TW20 0EX, England* (371)

MUTTAIYA SUNDARALINGAM, *Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706* (445)

HAMAO UMEZAWA, *Microbial Chemistry Research Foundation, Institute of Microbial Chemistry, 14-23, Kamiosaki 3-Chome, Shinagawa-ku, Tokyo, Japan* (183)

SUMIO UMEZAWA, *Department of Applied Chemistry, Faculty of Engineering, Keio University, 832 Hiyoshi, Kohoku-ku, Yokohama-shi, Japan* (111)

* Present address: Department of Chemistry, Queen Elizabeth College, University of London, Kensington, London W8 7AH, England.

PREFACE

The elucidation of structure of many complex, natural carbohydrates, especially polysaccharides and their conjugates, has progressed rapidly in the past few years, both in scope and precision, as a result of newer methods for specific degradation of these large molecules and for separating and identifying the products. In this thirtieth volume of *Advances*, Dutton (Vancouver) concludes his comprehensive treatment of published methodology for gas-liquid chromatography of sugars and their derivatives with the second part of a two-part Chapter; Part I was published in Volume 28. The present volume also features the first part of an article by Marshall (Miami) on the application of enzymic methods for structural analysis of polysaccharides.

Notable developments in the field of aminoglycosidic antibiotics have been recorded in recent years, especially by investigators in Japan. The medicinal scope of these therapeutic agents has been extended by useful semisynthetic variants, as well as by the discovery of new antibiotics produced microbially, and the rational design of effective, chemically modified agents has been guided by detailed biochemical investigations of the mode of action and of inactivation of these antibiotics. The outstanding team of Sumio Umezawa (Yokohama) and Hamao Umezawa (Tokyo) has been at the forefront of these advances, and these two investigators respectively contribute complementary Chapters on the structure and synthesis of the aminoglycosidic antibiotics, and on the biochemical mechanism of the development of resistance to them.

The disaccharide α,α -trehalose is the principal sugar of the circulatory system of insects, and its metabolism is discussed in a Chapter by Elbein (San Antonio). This subject has received relatively little study in comparison with the vast literature on metabolism of D-glucose in higher animals, but current interest in insect physiology and ecological problems associated with the use of traditional insecticides suggests that the time may be ripe for increased emphasis on the metabolism of α,α -trehalose. Sidebotham (London) contributes a survey of the extensive literature on the dextrans, microbial polysaccharides that are of considerable importance in several areas of applied biochemistry and technology, as well as being of fundamental interest.

This volume also presents the first of a projected, regular series of bibliographic articles that compile those carbohydrate structures that have been definitively established by crystallographic methods. Jeffrey (Pittsburgh) and Sundaralingam (Wisconsin) list here, with brief

descriptive details, those crystallographically determined structures of sugars, nucleosides, and nucleotides that were reported during 1970–1972; future volumes will contain similar articles for successive calendar years.

The obituary article by Bacon (Aberdeen) and Manners (Edinburgh) provides an interesting insight into the personality and career of David J. Bell.

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Kensington, Maryland
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R. STUART TIPSON
DEREK HORTON



David James Bell

1905-1972

DAVID JAMES BELL

1905-1972

David James Bell was born in Scotland on August 12, 1905, the only child of John J. and Helen Geddes Bell. His parents were living at Blairmore in Argyll, but, because there was no doctor there, his mother crossed the Firth of Clyde and he was born in Greenock. As a child, he looked eastward up the great river estuary to the city of Glasgow in which he was to receive his schooling and his university education; Glasgow had been created by the Clyde when, after the Act of Union with England in 1707, it became the chief port for the tobacco trade with the North American colonies. A century later, it was caught up in the industrial revolution, but, unlike the great industrial centres of Northern England, it had a university and a civic tradition when those places were little more than villages. When Bell graduated from the University of Glasgow in 1928, it was as the heir to a distinct Scottish culture, and, despite a long and scientifically profitable stay in Cambridge, he never lost his national characteristics and his affection for his native land.

Along the shores of the Clyde, the wealthier families of Glasgow had created summer resorts, of which Blairmore was one. Bell's father came from such a family (tobacco manufacturers whose name is still known to the pipe smoker) and had given up a science course at the university to risk a career in journalism and writing. By the turn of the century, he had become a popular and successful author, and is still remembered today for his Wee Macgreegor stories. His writings show a mastery of language, a preoccupation with verbal humour, and a sense of fun. Thus, in a pleasantly written travel book he could not resist describing the Clyde paddle steamers as "plying to capacity" and calling the Old Man of Storr, a rock pinnacle 50 meters high on the Isle of Skye, a "rude obelisk." A series of word pictures of a small boy on holiday with his father, "Jim Crow," published in 1910, was based on his son's early years. Echoes of the puns in it were still to be heard in laboratories fifty years later, and, without doubt, his son learnt much of his craft with words and his style of humour from his father.

Although Scotland's universities gave birth to and nourished the British school of carbohydrate chemistry, Glasgow University had least to do with it. Bell moved to St. Andrews in 1928, and took his Ph. D. under the supervision of J. C. Irvine. He investigated the chemistry of

the so-called α -celluloses of wood, namely, that fraction which is resistant to the action of 17.5% sodium hydroxide solution. The results were published in 1932 in the *Biochemical Journal*. The St. Andrews laboratory in this period is well described in the obituary notice of C. B. Purves [by A. S. Perlin, *Advan. Carbohydr. Chem.*, 23, 1 (1968)], whom he followed to J. J. R. Macleod's Department of Physiology in Aberdeen in 1931. This relatively brief period in St. Andrews had a determining effect on his research career, and, from this time on, he was never really happy unless he had at least one carbohydrate problem in hand. However, the subsequent years in Aberdeen were equally influential, and while continuing to make important contributions to carbohydrate chemistry, he had always an air of detachment from the intense activity then centred at the University of Birmingham.

Physically, Bell was a Scottish type, small in stature but well built. He had none of the dourness that some might have expected. He was a clear and fluent speaker, equally at home as a lecturer or a raconteur, and fond of social intercourse. There was a great deal of mischief in him, but little malice. His Scottish background gave him less respect for social status than that of the average Englishman; this went well in the laboratory, but less well with administrative hierarchies, which he could never take sufficiently seriously, his comments often being too witty and too near the truth for his own good.

To understand the form of his contribution to carbohydrate chemistry, two factors must be taken into account. The first is that, after leaving St. Andrews to go to Aberdeen in 1931, he was never again to work in a Department of Chemistry. He was, therefore, never to have the plentiful succession of Ph. D. students that most senior teaching-staff in chemistry could normally expect. In his whole career, he had only six research students.

Secondly, it has never been easy for outsiders to establish themselves in Cambridge. When he arrived in 1936, university salaries were not generous, being set in the expectation that a lecturer would earn additional money by teaching in one of the colleges. As required by statute, Bell was attached to a college (Jesus), but the authorities could find him no teaching to do; until 1935, biochemistry had been taught almost exclusively to medical students, and these were already catered for. He looked for work in other colleges, and it was in this way that one of us (J.S.D.B.) first met him in 1936. Whether from this early discouragement or from deliberate choice, Bell did not pursue a college career, but applied himself assiduously to research and teaching in the university department, activities that could lead to internal promotion, or, more usually, to a better teaching-post elsewhere.

The Cambridge Biochemical Laboratory under F. G. Hopkins was, by any standards, a remarkable place, and Bell made the fullest use of it. At Aberdeen, he had worked single-handed most of the time, except for his collaboration with H. W. Kosterlitz and F. G. Young, but, in pre-war Cambridge, his enthusiasm and, particularly, his willingness to work long hours at the bench won him the collaboration of staff (E. Baldwin), students (R. L. M. Synge, J. S. D. Bacon, J. Lorber), full-time research workers (M. Stephenson, E. Friedmann), and a senior technician (S. Williamson). During this period, the streak of restlessness in his character showed itself chiefly in daily wanderings (complete with pipe) round the Department—an important ingredient for the success of his collaboration—and in frequent moves from room to room. One of us (J.S.D.B.), who spent a total of five years with him, worked in four different rooms, and the other (D.J.M.) in two more. One detects a family influence, because he had lived with his parents in fifteen houses before he reached Cambridge at the age of 30.

When the Second World War broke out, he lost his Ph. D. students, and as the laboratory turned towards war work, he became increasingly restless and dissatisfied with his own contribution, and so he joined the Army, becoming a Major in the Royal Engineers. Here he found pleasure in a new circle of acquaintances, among whom was Rowland Hilder, a painter well known for his East Anglian landscapes. He was also intrigued to receive memoranda signed by Nigel Balchin, author of "The Small Back Room."

He was recalled to his teaching post in 1944 and at once settled back into laboratory work and publication, although, somewhat later (again in uniform), he went to occupied Germany with Professor John Beattie to investigate cases of famine edema. Later still, in 1947, he went, at the request of the Foreign Office, to teach in the medical school of the University of Münster-Westfalen. Thereafter, he was busy with the post-war "bulge" of university students and with the gradual return to more normal conditions in the early fifties. During this time, he collaborated with staff (G. D. Greville, D. H. Northcote), research students (Anne Palmer, D. J. Manners, Nancy E. Hardwick), and workers in other laboratories, including J. Barcroft, P. H. Blanchard, R. Dedonder, J. Edelman, D. Gross, and F. A. Isherwood.

It was during his stays in Aberdeen and Cambridge, spanning the years 1931–1954, that he made his chief contributions to carbohydrate chemistry, and for these he was awarded the degree of Sc. D. by Cambridge in 1950.

In Aberdeen, he was first a Carnegie Teaching Fellow and then an 1851 Exhibition Senior Student in the Department of Physiology. Under

Macleod's influence, he showed that the low optical activities of blood filtrates and dialyzates were not due to the presence of D-glucofuranose, examined the action of adrenaline on the hexose monophosphate content of rat muscle, and began his researches on glycogen, a polysaccharide that was to become a continuing and major interest during the next 20 years. With F. G. Young, he critically examined the criteria for the isolation and purity of glycogen, and, with H. W. Kosterlitz, he studied the properties of acetyl and benzoyl derivatives of glycogen, and of the de-acylated polysaccharide esters. He carried out the first methylation analysis of fish-liver glycogen: the average chain-length of this and of a sample of rabbit-liver glycogen was found to be 12.

Bell's major contribution to carbohydrate chemistry was undoubtedly in the development of methods for the preparation of numerous methylated sugars, particularly the methyl ethers of D-glucose, D-galactose, and, later, D-fructose, and for their quantitative separation. This work also began in Aberdeen, where syntheses of methyl 2,3,6-tri-O-benzoyl- α -D-glucoside, 3,6-di-O-methyl-D-glucose, and 6-O-methyl-D-glucose were performed, the removal of the dichloroacetyl group from various D-glucose derivatives and the reaction of acetone with methyl 2-O-methyl-D-glucofuranoside were examined, and the preparation of 1,2:5,6-di-O-isopropylidene- α -D-glucofuranose was improved.

At Cambridge, these two major lines of research—on methylated sugars and on glycogen—were developed, and Bell's interest in other polysaccharides was expanded. This period saw the preparation, either for the first time, or in an improved form, of 3,6-, 4,6-, and 2,6-di-O-methyl-D-glucose, and 2-, 2,6- and 3,4-di-, and 2,4,6-tri-O-methyl-D-galactose. A number of reactions were examined, including that between *p*-tolylsulfonyl derivatives of D-glucofuranose and sodium iodide, acyl migration in the D-galactose series, and the use of the benzyl group in syntheses of methylated sugars. On the analytical side, he developed a micro-method for the determination of the isopropylidene group in carbohydrate derivatives, and examined the relationship between refractive index and specific rotation in various methyl D-galactosides.

Substantial progress was also made with glycogen. Whereas the results of previous methylation analysis had indicated average chain-lengths of 12 for rabbit-liver glycogen [compare also, W. N. Haworth and E. G. V. Percival, *J. Chem. Soc.*, 2277 (1932)], glycogen formed in rabbit livers after the ingestion of D-galactose was found to have an average chain-length of 18. A similar value was independently obtained by W. N. Haworth, E. L. Hirst, and F. A. Isherwood [*J. Chem. Soc.*, 577 (1937)] for a sample of muscle glycogen. This result emphasized that metabolic reserves of carbohydrate are not chemically static, but that their overall

structure reflects the state of the tissue at the time of isolation of the polysaccharide. Methylation analyses were also performed on glycogen from *Mytilus edulis*, horse muscle, and *Helix pomatia*. Interest in the latter invertebrate also led to an examination of snail galactogen (galactan), which revealed that both D- and L-galactose are present therein.

R. L. M. Synge, who began research in 1936 with N. W. Pirie's suggestion that he should investigate the chemistry of glycoproteins, sought Bell's help and published his first papers with him. Recalling this in his Nobel Lecture, he said, "Among many useful things, I learnt from Bell the power of liquid-liquid extraction, with or without salting out, for separating methylated sugars according to the extent of their methylation." Synge and Martin's first papers on partition chromatography appeared in 1941, while Bell was in the Army, but, immediately upon his return to Cambridge in 1944, he devised and published a small-scale method for the separation of methylated glucoses on a column of silica gel which he applied successfully to the methylation analysis of rice starch, and of glycogen from horse muscle and *Ascaris lumbricoides*. In 1949, a similar method was developed for the separation of the methyl ethers of D-fructose, and was used for methylation analysis of fructans from higher plants and bacteria. Similar methods were used for the characterization of the oligosaccharides produced from sucrose by various invertase preparations. Synge ["Les Prix Nobel en 1952," P. A. Norstedt & Soner, Stockholm, pp. 122-135] commented: "Partition chromatography has proved almost ideally adapted for analysing the hydrolysis products of methylated polysaccharides, . . . greatly increasing the scope of the methylation method . . . It gives me great pleasure that my teacher D. J. Bell was the first to use the method for this purpose."

In the post-war period, Bell characterized 2,6- and 2,3-di-O-methyl-D-galactose and 3,4-di-O-methyl-D-glucose, prepared 2,4-di-O-methyl-D-glucose for the first time, and examined the periodate oxidation of several methylated sugars. Studies on glycogen continued, with an examination of the liver glycogen from rabbits that had been fed with D-glucose, D-fructose, and sucrose, and of the molecular weights of several samples of glycogen by sedimentation-diffusion methods. The nature of the inter-chain linkages in glycogen had been the subject of some controversy, and these were finally characterized as (1→6)- α -D-glucosidic by periodate oxidation. A study of the *beta*-amylolysis of glycogen provided information on the relative lengths of the external and internal chains. Many of these results were reported in an excellent review [*Biol. Rev.*, 23, 256 (1948)].

Other studies in this post-war period dealt with the presence of D-fructose in fetal blood, the renal clearances of some fructans in the

dog, the structures of yeast glucan and of the cellulose from *Posidonia australis*, and the presence of D(+)-apiose in the same plant-tissue. He also wrote reviews on enzymic, *in vitro* syntheses of natural glycosidic derivatives [*Ann. Repts. Chem. Soc.*, 44, 217 (1948)], on carbohydrate chemistry [*Ann. Rev. Biochem.*, 18, 87 (1949)], on the methyl ethers of D-galactose [*Advan. Carbohydr. Chem.*, 6, 11 (1951)], and on mono- and oligo-saccharides and acidic monosaccharide derivatives [*Modern Methods of Plant Analysis*, Vol. II, 1 (1955)].

Through all this period, he personally carried out a large proportion of the experimental work. His early training in St. Andrews had ensured that his glass-blowing skill was an example to others, and he had an uncanny ability in being able to crystallize the most intractable of carbohydrate syrups. His knowledge of the literature, particularly that of the 1920-30 period, was extensive. He shared these characteristics with his friend and former colleague, Dr. J. W. H. Oldham. Bell was the author or co-author of some 100 publications, constituting ample testimony to his industry and success in the laboratory.

In the University of Cambridge, David Bell's influence extended far beyond his own laboratory. He excelled as a lecturer to the large undergraduate classes in Part I biochemistry and physiological chemistry. In the advanced classes, his lectures had an air of quiet authority. These lectures were enlivened by humorous anecdotes, usually slightly exaggerated, concerning some personality from the literature, or stories from the St. Andrews' laboratory, where hydrolyses of polysaccharides were apparently carried out while the chemists concerned (including Bell) were otherwise occupied on the nearby golf-course! He was the author of an "Introduction to Carbohydrate Biochemistry" which was produced in three editions between 1940 and 1952, and, with his great friend Ernest Baldwin, he revised, for the benefit of the medical classes, Cole's "Practical Physiological Chemistry."

During the early fifties, his longing to return to Scotland intensified, and he eventually achieved this in 1954 by abandoning teaching, a step that had seemed an impossibility to his friends, and taking a full-time research post in the Poultry Research Centre at Edinburgh, one of the Institutes of the Agricultural Research Council. Here, he began to investigate various aspects of the metabolism of the domestic fowl, in particular, the nitrogen-containing metabolites, and the enzyme levels in various tissue-fluids. It is a measure of his wide range of interests, his breadth of knowledge, and his enthusiasm for experimental work, that, during the next 11 years, he was the author of 21 publications devoted to this area of physiology and biochemistry. His collaborators in this period included I. E. Lush (his fifth Ph. D. student), P. E. Lake, T. P.

Bird, J. G. Campbell, J. Culbert, W. M. McIndoe, and W. G. Siller. He also edited, with B. M. Freeman, a three-volume treatise on the "Physiology and Biochemistry of the Domestic Fowl" (Academic Press, New York, 1971), accepted as the major authoritative work on the subject. He retained some contact with undergraduates by serving as an honorary, senior lecturer in the Department of Physiology in the University of Edinburgh, and, in 1965, he left the institute to become a full-time member of that department.

Once again, he was able to devote himself wholeheartedly to his beloved carbohydrates. He examined the transport of monosaccharides within certain tissues, and their excretion in urine. Here, his chemical expertise was used to develop appropriate, analytical techniques, and, with M. Q. K. Talukder (his last Ph. D. student), he devised a thin-layer chromatographic technique for estimating pentoses in the presence of other sugars. At a time when many of his contemporaries and junior colleagues were "desk-bound," Bell was still carrying out experiments himself, handling minute amounts of sugars with the complete confidence that had developed from years of experience. In 1970, he officially retired, but he continued in the department, both as a teacher and a research worker, with the aid of grants from the Medical Research Council and from the British Diabetic Association.

During his years in Edinburgh, he wrote a comprehensive account of the "Structure and occurrence of natural monosaccharides and oligosaccharides" [*Comparative Biochemistry*, 3A, 288 (1962)] and an essay for sixth-formers on the energetics of biochemical processes for "Four Aspects of Energetics" [by A. Finch and others, Wm. Collins Sons & Co. Ltd., London, 1970], which reads like a transcript of his very lucid, extempore lecturing-style.

Within the United Kingdom, Bell was well known amongst carbohydrate chemists. He was a meticulous examiner of Ph. D. theses, as some candidates found to their dismay, and an active member of the British Carbohydrate Nomenclature Committee, where he had a particular interest in branched-chain sugars. He served as a member of the Publications Committee of The Chemical Society from 1951–1955, and as Senior Reporter for the biochemistry section of the *Annual Reports of The Chemical Society*. He frequently travelled to Europe, notably to the foreign meetings of the Society for Experimental Biology, and he particularly valued his contacts with the carbohydrate biochemists at the Institut Pasteur.

In 1950, his first wife, Joan Collie, whom he had married in Aberdeen, died after a lengthy illness, and, in 1951, he married June Cross, a medically qualified biochemist. He is survived by his widow and their two

daughters, and by a son and daughter and three grandsons from his first marriage.

His return to Scotland had been marked by his election to the Royal Society of Edinburgh, and by renewed contacts with many friends within and without the Scottish universities. It was a special pleasure for him to be within walking distance of Sir Edmund Hirst's department. He settled close to the centre of Edinburgh with his young family, became active in the Episcopalian Church, and was able to indulge his enthusiasm for golf again. His friends saw little change in him from his Cambridge days, and so it was a shock to all when he died suddenly, of a heart attack, on April 3rd, 1972.

Those who have had the privilege of working with him have always found it difficult to explain fully the particular pleasure of his company at the bench. The intent was serious, but the execution was often light-hearted, extending even to the labels on the bottles. Where else in the world was there a *M/100* cockroach solution? Recently, one of us asked the other "What was Bell's reagent?" Neither of us can really remember, but one thing is certain—that without it on the shelf, our laboratories are duller places.

JOHN S. D. BACON
DAVID J. MANNERS

APPLICATIONS OF GAS-LIQUID CHROMATOGRAPHY TO CARBOHYDRATES*: PART II**

BY GUY G. S. DUTTON

*Department of Chemistry, The University of British Columbia,
Vancouver V6T 1W5, British Columbia, Canada*

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INTRODUCTION

This second part of a two-part article is concerned with the separation of methylated sugars and their derivatives by gas-liquid chromatography (g.l.c.). The first subjection of carbohydrates to g.l.c. was as their methyl derivatives,¹ and these applications have been reviewed.²⁻⁴ The present article is concerned only with papers published since 1964. In addition to those references already cited in the introduction to Part I, Volume 6 of *Methods in Carbohydrate Chemistry*⁷³² contains a section on the separation of methylated sugars by g.l.c.

As methylated sugars are normally encountered in work concerned with the determination of the structure of di- to poly-saccharides, the present account commences with a brief survey of methods of methylation before passing on to the different ways by which methylated sugars may be separated by using g.l.c.

It is worth noting that, almost simultaneously, three separate techniques have been developed, each of which has simplified the determination of polysaccharide structures; in combination, these three methods have revolutionized structural investigations. They are (*a*) improved methods of methylation, (*b*) the separation by g.l.c. of mixtures of methylated sugars, and (*c*) the identification of these separated compounds by mass spectrometry. The last topic is treated only briefly in this article, as it is discussed in detail elsewhere in this Series.⁷³³ Lindberg and coworkers have reviewed these three associated developments.⁷³⁴

XVI. METHODS OF METHYLATION

The method of determining di- and poly-saccharide structures by methylation has been used since the beginning of the century, and is traditionally associated with the names of Purdie and Irvine⁷³⁵ and of

(732) H. G. Jones, *Methods Carbohydr. Chem.*, **6**, 25 (1972).

(733) J. Lönngren and S. Svensson, *Advan. Carbohydr. Chem. Biochem.*, **29**, 41-106 (1973).

(734) H. Björndal, C. G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem.*, **82**, 643 (1970); *Angew. Chem. Int. Ed. Engl.*, **9**, 610 (1970).

(735) T. Purdie and J. C. Irvine, *J. Chem. Soc.*, **83**, 1021 (1903).

Haworth.⁷³⁶ These methods remained unchanged for many years, until certain aprotic solvents became commercially available. The first major advance was the use by Kuhn and his associates of *N,N*-dimethylformamide or methyl sulfoxide (Me_2SO) as solvents in conjunction with methyl iodide or methyl sulfate, and silver oxide or strontium oxide,^{737,738} and, at about the same time, Roth and Pigman^{738a} used methyl sulfoxide for the methylation of amino sugars.

Although reaction in *N,N*-dimethylformamide greatly facilitates methylation, the method suffers from the limitation that it often gives low recoveries of methylated polysaccharides,³⁹² and the effectiveness of the methylation appears to depend on the volume of *N,N*-dimethylformamide used.⁷³⁹ Even with monosaccharides, incomplete methylation has been reported.⁷⁴⁰ D. A. Rees and coworkers have given details of a modified Kuhn methylation for carrageenan,⁷⁴¹ and they also described the methylation of dextran⁷² and of a xylan from red seaweed in *N*-methyl-2-pyrrolidinone.⁷⁴² A micro Kuhn method suitable for oligosaccharides has been described by Perila and Bishop.⁷⁴³

In applying the Kuhn methylation to a pentasaccharide isolated from amylopectin α -limit dextrin, the maximum methoxyl content that French, E. E. Smith, and Whelan⁷⁴⁴ were able to obtain was 41.8% (theoretical, 49.5%). Quantitative analysis of the hydrolyzate showed that there was no undermethylation, and they concluded that it is essential to wash the product with cyanide after each methylation in order to eliminate chloroform-soluble impurities containing iodine. Kabat and coworkers⁷¹⁶ found that a Kuhn methylation of hexosamines using silver oxide did not give interpretable results, but no problem was experienced when barium oxide was employed.

Srivastava and coworkers published a method of methylation in methyl sulfoxide with barium oxide and methyl iodide,⁷⁴⁵ but they later found

(736) W. N. Haworth, *J. Chem. Soc.*, **107**, 8 (1915).

(737) H. Egge, R. Brossmer, A. Gauhe, P. Klesse, W. Lochinger, E. Rohm, H. Trischmann, and D. Tschampel, *Angew. Chem.*, **72**, 805 (1960).

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(738a) W. Roth and W. Pigman, *J. Amer. Chem. Soc.*, **82**, 4608 (1960).

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(740) B. Flaherty, W. G. Overend, and N. R. Williams, *J. Chem. Soc.*, 398 (1966).

(741) T. C. S. Dolan and D. A. Rees, *J. Chem. Soc.*, 3534 (1965).

(742) A. S. Cerezo, A. Lezerovich, R. Labriola, and D. A. Rees, *Carbohydr. Res.*, **19**, 289 (1971).

(743) O. Perila and C. T. Bishop, *Can. J. Chem.*, **39**, 815 (1961).

(744) D. French, E. E. Smith, and W. J. Whelan, *Carbohydr. Res.*, **22**, 123 (1972).

(745) H. C. Srivastava, S. N. Harshe, and P. P. Singh, *Tetrahedron Lett.*, 1869 (1963).

that better yields were obtained by using methyl sulfate and powdered sodium hydroxide.⁷⁴⁶ The former reagents have been particularly recommended for polysaccharides rich in hexosamines^{747,748} and the two methods have been used to methylate dextrans,⁷⁴⁹ a galactofucomannan,⁷⁵⁰ tritylated arabinogalactans,⁷⁵¹ xylans,^{673,752,753,753a} an amyloid,⁷⁵⁴ a galactan,⁷⁵⁵ and heteropolysaccharides.^{332,677}

Occasionally, methylations are conducted in liquid ammonia, and typical examples are to be found in papers on *Xanthomonas campestris*⁷⁵⁶ and glucans.^{757,757a}

The most important development in methylation technique is due to Hakomori,⁷⁵⁸ who used as the base methylsulfinyl carbanion, formed by reaction of methyl sulfoxide with sodium hydride, and thus extended to carbohydrates the reaction developed by Corey and Chaykovsky.⁷⁵⁹ In certain cases, completely methylated polysaccharide may be obtained in high yield by one treatment within an hour. This method was used by Sandford and Conrad in a re-examination of the polysaccharide from *Aerobacter aerogenes*, and their paper should be consulted for the excellent experimental details contained therein.⁷⁶⁰ These authors showed that this polysaccharide has a simple, repeating structure, and previous suggestions that it is complicated and highly branched arose from interpretation based on products of incomplete methylation.

The Hakomori method has, within a short time, been applied to poly-

- (746) H. C. Srivastava, P. P. Singh, S. N. Harshe, and K. Virk, *Tetrahedron Lett.*, 493 (1964).
- (747) E. Moczar and L. Mester, *Bull. Soc. Chim. Biol.*, **46**, 881 (1964).
- (748) S. A. Barker, S. M. Bick, J. S. Brimacombe, M. J. How, and M. Stacey, *Carbohydr. Res.*, **2**, 224 (1966).
- (749) B. Lindberg and S. Svensson, *Acta Chem. Scand.*, **22**, 1907 (1968).
- (750) R. N. Fraser and B. Lindberg, *Carbohydr. Res.*, **4**, 12 (1967).
- (751) G. O. Aspinall, R. M. Fairweather, and T. M. Wood, *J. Chem. Soc. (C)*, 2174 (1968).
- (752) Š. Karácsonyi, M. Kubačková, and J. Hrivnák, *Collect. Czech. Chem. Commun.*, **32**, 3597 (1967).
- (753) N. W. H. Cheetham and R. J. McIlroy, *Carbohydr. Res.*, **11**, 187 (1969).
- (753a) N. W. H. Cheetham and R. J. McIlroy, *Carbohydr. Res.*, **21**, 201 (1972).
- (754) S. E. B. Gould, D. A. Rees, and N. J. Wight, *Biochem. J.*, **124**, 47 (1971).
- (755) P. J. Wood and I. R. Siddiqui, *Carbohydr. Res.*, **22**, 212 (1972).
- (756) I. R. Siddiqui, *Carbohydr. Res.*, **4**, 283 (1967).
- (757) D. M. Bush and M. Horisberger, *Carbohydr. Res.*, **22**, 361 (1972).
- (757a) E. J. Bourne, R. L. Sidebotham, and H. Weigel, *Carbohydr. Res.*, **22**, 13 (1972).
- (758) S. Hakomori, *J. Biochem. (Tokyo)*, **55**, 205 (1964).
- (759) E. J. Corey and M. Chaykovsky, *J. Amer. Chem. Soc.*, **84**, 866 (1962).
- (760) P. A. Sandford and H. E. Conrad, *Biochemistry*, **5**, 1508 (1966).

saccharides of many different types, and has, in virtually all instances, given excellent results. Representative applications are given in Table XIV (see p. 53). There are a few reports where the method was found inapplicable, due to the insolubility of the polysaccharide in the solvent.^{590,761,762} It is probable that this insolubility was attributable to incomplete de-ionization of the sample, a situation that may be encountered with polysaccharides containing acid groups (for example, glucuronoxylans) or where inorganic complexes have been used in the isolation procedure (for example, glucomannans precipitated by barium hydroxide). In our laboratory, crude, bacterial polysaccharides and hemicelluloses found quite insoluble in methyl sulfoxide dissolved readily after careful de-ionization. Similarly, a phosphorylated lipopolysaccharide was insoluble in methyl sulfoxide, but could, with some difficulty, be methylated after acetylation. By contrast, the dephosphorylated polysaccharide was freely soluble, and was fully methylated by one treatment.⁷²⁹ When a Hakomori methylation has been preceded by a Haworth methylation in order to obtain solubility in methyl sulfoxide, the procedure has been called the Unrau method.⁶⁹⁹

When methylsulfinyl carbanion is added to a solution of polysaccharide in methyl sulfoxide, a gel is often formed, and methylation may be aided by ultrasonic agitation.^{444,445,678,763-765}

The Hakomori method of methylation may be applied to mono- or poly-saccharides, and detailed procedures are available.^{444,760,764,766} Methylation in methyl sulfoxide appears to be similar to the method of Kuhn in that furanoside formation at the reducing group is favored,⁷⁶⁷ and, where this is undesirable, preliminary methylation with diazomethane is recommended.⁷⁶⁸

D. M. W. Anderson and Cree⁷⁶⁹ reported the successful methylation of a variety of polysaccharides with sodium hydride in methyl sulfoxide, and their method has been used by others.^{718,770} Alkylations have also

(761) J. S. G. Reid and K. C. B. Wilkie, *Phytochemistry*, **8**, 2053 (1969).

(762) G. G. S. Dutton and N. A. Funnell, unpublished results.

(763) K. Axelsson, H. Björndal, and K.-E. Eriksson, *Acta Chem. Scand.*, **22**, 1363 (1968).

(764) C. G. Hellerqvist, B. Lindberg, and K. Samuelsson, *Acta Chem. Scand.*, **22**, 2736 (1968).

(765) O. Larm, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **20**, 39 (1971).

(766) H. E. Conrad, *Methods Carbohydr. Chem.*, **6**, 361 (1972).

(767) J. M. Berry and G. G. S. Dutton, *Can. J. Chem.*, **50**, 1424 (1972).

(768) M. E. Gelpi, J. O. Deferrari, and R. A. Cadenas, *J. Chem. Soc. (C)*, 3354 (1971).

(769) D. M. W. Anderson and G. M. Cree, *Carbohydr. Res.*, **2**, 162 (1966).

(770) E. Percival, *Carbohydr. Res.*, **17**, 121 (1971).

been conducted by using sodium hydride in *N,N*-dimethylformamide.⁷⁷¹⁻⁷⁷³

Although such a powerful base as methylsulfinyl carbanion might be expected to cause degradation of polysaccharides by β -elimination, especially when uronic acids are present,⁷⁷⁴ occurrence of degradation has been denied.⁷⁷⁵ However, the same authors⁷⁷⁵ showed that, when a rhamnosyl group is attached to O-4 of glucuronic acid, a fair proportion of such groups is lost during methylation. Arabinofuranosyl groups have also been reported lost by conventional methylation,⁷⁷⁶ and the same applies to galactofuranosyl groups⁶⁷⁴ and xylosyl groups.⁷⁷⁰ Similarly, methylation of glycosiduronic acids has been shown to afford a small proportion of the 4,5-alkenic compounds.^{538,777} Perlin and coworkers⁷⁷⁸ have drawn attention to facile eliminations that accompany some oxidations with methyl sulfoxide, and several groups of authors have discussed β -elimination reactions of methylated uronic acid derivatives.⁷⁷⁹⁻⁷⁸¹ Because of the possibility of β -elimination in acidic polysaccharides during a Hakomori methylation, if complete substitution is not achieved by one treatment, remethylation should be performed by an *alternative* procedure, or a fresh sample should be taken.⁷³⁴ For these, and other, reasons it may be convenient, or desirable, to reduce uronic acids before methylation.^{26,467,781a} When the neutral monosaccharide corresponding to the uronic acid is already a constituent of the polysaccharide, reduction may be performed by use of deuterated^{484a,781b} or tritiated reagents,^{781c} thus permitting distinction of products by mass spectrometry or by radioactive counting.

- (771) J. S. Brimacombe, B. D. Jones, M. Stacey, and J. J. Willard, *Carbohydr. Res.*, **2**, 167 (1966).
- (772) M. E. Tate and C. T. Bishop, *Can. J. Chem.*, **41**, 1801 (1963).
- (773) P. J. Brennan, D. P. Lehane, and D. W. Thomas, *Eur. J. Biochem.*, **13**, 117 (1970).
- (774) K. Hunt and J. K. N. Jones, *Can. J. Chem.*, **40**, 1266 (1962).
- (775) D. M. W. Anderson, I. C. M. Dea, P. A. Maggs, and A. C. Munro, *Carbohydr. Res.*, **5**, 489 (1967).
- (776) A. M. Stephen, M. Kaplan, G. L. Taylor, and E. C. Leisegang, *Tetrahedron*, Suppl. 7, 233 (1966).
- (777) P. Kováč, *Carbohydr. Res.*, **22**, 464 (1972).
- (778) G. M. Cree, D. W. Mackie, and A. S. Perlin, *Can. J. Chem.*, **47**, 511 (1969).
- (779) J. Kiss, *Advan. Carbohydr. Chem. Biochem.*, **29**, 229-303 (1973).
- (780) G. O. Aspinall and P. E. Barron, *Can. J. Chem.*, **50**, 2203 (1971).
- (781) H. Nashimoto, T. Sekiyama, H. Sakai, and J. Yoshimura, *Bull. Chem. Soc. Jap.*, **44**, 235 (1971).
- (781a) M. Okigawa, H. Hatanaka, N. Kawano, I. Matsunaga, and Z. Tamura, *Tetrahedron Lett.*, 2935 (1970).
- (781b) K. Axelsson and H. Björndal, *Acta Chem. Scand.*, **24**, 713 (1970).
- (781c) D. A. Rees and N. J. Wight, *Biochem. J.*, **115**, 431 (1969).

An interesting paper by Handa and R. Montgomery⁷⁸² compared the results of partially methylating methyl α -D-mannopyranoside by the methods of Haworth, Kuhn, and Hakomori. Considerable difference was found in the reactivity of the various hydroxyl groups according to the method used. Thus, the hydroxyl group at C-6 was found the most reactive under Haworth conditions, the least reactive with the Kuhn reagents, and intermediate in the Hakomori method. These results suggest the desirability of using various methylation techniques when difficulty is encountered in obtaining a fully methylated polysaccharide. Aspinall and Fairweather⁷⁸³ suggested that the problem of permethylating polysaccharides that contain galactose is associated with the axial hydroxyl group at C-4. Timell and coworkers⁷⁷¹ reported difficulty in obtaining fully methylated products from compression-wood galactan, and gave references to similar cases. Sulfated polysaccharides are also difficult to methylate completely.⁷⁸⁴

Cheetham and McIlroy⁷⁵³ compared the efficiency of methylating xylans by the Haworth, the Hakomori, and the Srivastava procedures. They concluded that the Hakomori method readily gives a high degree of methylation with the minimum amount of degradation, as judged by vapor-phase osmometry of the products. This paper also gave useful experimental details of the methylation procedure. Other authors have reported a lowering of the degree of polymerization (d.p.) in the methylation of pectins.^{785,786}

All of the studies just cited were concerned with methylation. Saier and Ballou⁶⁵⁴ reported that *Mycobacterium phlei* produces a lipopolysaccharide that contains 3-O- and 6-O-methyl-D-glucose residues. In investigating the structure of such a material, clearly methylation is not applicable. They⁶⁵⁴ tried ethylation as an alternative, but found that the resulting glycosides were not separable by g.l.c., although partial separation of the free sugars was obtained by thin-layer chromatography (t.l.c.). It was subsequently shown that propylation was satisfactory and permitted the separation by g.l.c. of methyl 6-O-methyl-2,3,4-tri-O-propyl-D-glucosides from methyl 3-O-methyl-2,4,6-tri-O-propyl-D-glucosides.

An alternative approach was used by Gray and Ballou⁷⁰⁰ to study a polysaccharide, containing 3-O-methyl-D-mannose and D-mannose, from *Mycobacterium phlei*. When the mycobacteria were grown on (methyl-

(782) N. Handa and R. Montgomery, *Carbohydr. Res.*, **11**, 467 (1969).

(783) G. O. Aspinall and R. M. Fairweather, *Carbohydr. Res.*, **1**, 83 (1965).

(784) J. R. Nunn and H. Parolis, *Carbohydr. Res.*, **6**, 1 (1968).

(785) C. J. B. Smit and E. F. Bryant, *J. Food Sci.*, **34**, 191 (1969).

(786) R. G. Ovodova and Yu. S. Ovodov, *Carbohydr. Res.*, **10**, 387 (1969).

¹⁴C)-methionine, the label was incorporated only in the methyl group of 3-O-methyl-D-mannose. Following ordinary methylation, radioactive counting permitted distinguishing those ethers originating from D-mannose and those from 3-O-methyl-D-mannose. The same two sugars occur in the lipopolysaccharide of *Klebsiella* O group 5, and Lindberg and co-workers⁷⁸⁷ distinguished between them by methylating with trideuterio-methyl iodide (CD₃I) and examining by mass spectrometry the methyl ethers obtained. The same authors⁷¹³ likewise used ordinary methylation, partial hydrolysis, and deuteriomethylation to study the distribution of the pyranoid and furanoid forms of the D-galactosyl residues in the lipopolysaccharide of *Klebsiella* O group 9. A similar method was used to study the distribution of acetate in a polysaccharide from *Rhizobium meliloti*.⁴⁴⁵ A combination of methylation and ethylation was used to study the length of the side chains in a dextran.⁷⁸⁵

Fully methylated polysaccharides should be purified by fractional extraction, which (a) is more satisfactory than fractional precipitation, and (b) provides a further check on the homogeneity of the sample.⁷⁸⁸ Gel chromatography on Sephadex LH-20 is an excellent way of purifying methylated polysaccharides,^{728,789} and Sephadex G-25 has been used to remove salts after a Kuhn methylation.⁷⁴⁷ Methylated mannans have also been purified on columns of silicic acid.⁷⁹⁰ Many authors consider that methylation is complete when the i.r. spectrum of the product ceases to show absorption for hydroxyl group, but the importance of performing methoxyl determinations is illustrated by the work of Lee and Ballou,⁷³⁹ who found that such a product still gave significant amounts of undermethylated compounds on methanolysis. Conversely, noncarbohydrate impurities may lower the methoxyl content.^{744,757a} The general principles of end-group analysis of polysaccharides have been reviewed⁷⁹¹ and, for succinct accounts of the errors that may arise from undermethylation, the papers of Wallenfels and coworkers⁷⁹² and Sandford and Conrad⁷⁶⁰ should be consulted.

The methylated polysaccharide must be methanolized or hydrolyzed under conditions that minimize demethylation, a problem to which reference has already been made.¹⁰² The sensitivity of analytical methods

- (787) B. Lindberg, J. Lönngren, and W. Nimmich, *Acta Chem. Scand.*, **26**, 2231 (1972).
- (788) E. L. Hirst and E. Percival, *Methods Carbohydr. Chem.*, **5**, 287 (1965).
- (789) M. H. Saier, Jr., and C. E. Ballou, *J. Biol. Chem.*, **243**, 4319 (1968).
- (790) R. J. Yu, C. T. Bishop, F. P. Cooper, H. F. Hasenclever, and F. Blank, *Can. J. Chem.*, **45**, 2205 (1967).
- (791) F. Smith and R. Montgomery, *Methods Biochem. Anal.*, **3**, 153 (1956).
- (792) K. Wallenfels, G. Bechtler, R. Kuhn, H. Trischmann, and H. Egge, *Angew. Chem.*, **75**, 1014; *Angew. Chem. Int. Ed. Engl.*, **2**, 515 (1963).

based on g.l.c. serves only to accentuate these problems. A study⁷⁹³ on wood glucomannans showed that significantly different values for tetra- and di-*O*-methyl sugars were obtained when one half of a methylated sample was hydrolyzed with formic acid and the other half with sulfuric acid.

Methylation analysis depends on determining accurately the ratio of fully methylated terminal units to those partially methylated units arising from interior portions of the molecule. It has long been recognized that fully methylated monosaccharides, particularly as their methyl glycosides, are significantly volatile, and that manipulative losses of such compounds introduce grave errors in quantitative results. For this reason, and particularly when large volumes of aqueous solutions have to be evaporated (as when ion-exchange resins are used), some workers have preferred to extract fully methylated glycosides with organic solvents^{429,793a} prior to concentration, and others⁶⁷⁴ have removed water azeotropically with ethanol at atmospheric pressure. Few quantitative studies have been made of the possible losses of such compounds during concentration, and the results of Timell and coworkers are of especial interest in this connection.^{599,600} They showed that, with methyl 2,3,4-tri-*O*-methyl-*D*-xyloside, 67% was lost by concentrating an aqueous solution at 50°/50 torr. When a chloroform solution of the same compound was concentrated by means of a stream of air at 20°, a loss of 99% occurred. The corresponding figures for methyl 2,3,4,6-tetra-*O*-methyl-*D*-glucoside were 15 and 95%. Concentration of the methylated monosaccharides by either method gave no loss in the case of the methylated *D*-glucose, but 8 and 43% loss for the methylated *D*-xylose. Arendt and Pacsu⁷⁹⁴ showed that, at room temperature, 19.2% of a sample of methyl 2,3,4,6-tetra-*O*-methyl- β -*D*-glucoside evaporated during 95 hours; they suggested that the volatility of this compound contributes to the problem of estimating the number of nonreducing end-groups in cellulose. Ballou and coworkers also commented on the loss of methyl 2,3,4,6-tetra-*O*-methyl-mannoside and -glucoside.^{794a} The removal of methanol and hydrogen chloride in a desiccator (not evacuated) containing calcium chloride and potassium hydroxide was recommended.^{794a} Other workers have noted the volatility of fully methylated compounds.^{429,795,796}

(793) G. G. S. Dutton and R. H. Walker, *Cell. Chem. Technol.*, **6**, 295 (1972).

(793a) G. G. S. Dutton and K. Hunt, *J. Amer. Chem. Soc.*, **82**, 1682 (1960).

(794) V. D. Arendt and E. Pacsu, *Text. Res. J.*, **35**, 554 (1965).

(794a) T. S. Stewart, P. B. Mendershausen, and C. E. Ballou, *Biochemistry*, **7**, 1843 (1968).

(795) J. Rosík, V. Zitko, Š. Bauer, and J. Kubala, *Collect. Czech. Chem. Commun.*, **31**, 3353 (1966).

(796) J. Rosík, M. Bruteničová-Sósková, V. Zitko, and J. Kubala, *Collect. Czech. Chem. Commun.*, **31**, 3410 (1966).

The observations on the effect of methods of hydrolysis^{102,793} and those concerning undermethylation^{760,792} make it clear that, despite significant improvements in technique, the results of methylation end-group analysis are incapable of exact interpretation when the degree of branching is low.^{793,797}

XVII. MODEL STUDIES ON METHYLATED SUGARS

The reviews by Bishop^{3,4} and H. G. Jones⁷³² gave tabular data on the retention times of methyl glycosides and other derivatives. Some papers concerned with the structure of carbohydrate materials provide much useful information on certain groups of compounds, and others examine model systems for the separation of a given class of sugar. This Section deals mainly with the latter type of study, and applications of these methods to structural problems will be discussed subsequently.

1. Aldopentoses

J. K. N. Jones and coworkers studied the separation of the methyl ethers of L-arabinose as their acetylated methyl glycosides and their acetylated alditols;⁷⁹⁸ they also reported paper and thin-layer chromatographic separations. In a similar investigation on the methyl ethers of D-xylose, separation as the alditol acetates, acetylated diethyl dithioacetals, and acetylated nitriles was examined.⁷⁹⁹ The acetylated nitriles were found to give sharp, single peaks that clearly distinguished all of the isomers used, including the 2- and 3-methyl ethers which, as the corresponding alditol acetates, have the same retention time.

The separation of methyl xylofuranosides on several different columns has been examined by Anderle and coworkers.^{800,801} In the first study,⁸⁰⁰ 3% of ECNSS-M was recommended as giving the best results, but it was subsequently found⁸⁰¹ that this column is incapable of separating methyl 2- and 3-O-methyl-D-xylofuranosides, and a column of 5% of XE-60 operated isothermally was found preferable. Kováč described syntheses of the methylated furanosides of 5-O-methyl-, 2,5- and 3,5-di-O-methyl-, and 2,3,5-tri-O-methyl-D-xylose.⁸⁰² An investigation⁸⁰³ into the arabinan

(797) G. C. Hoffmann, B. W. Simson, and T. E. Timell, *Carbohydr. Res.*, **20**, 185 (1971).

(798) S. C. Williams and J. K. N. Jones, *Can. J. Chem.*, **45**, 275 (1967).

(799) F. G. Lance and J. K. N. Jones, *Can. J. Chem.*, **45**, 1995 (1967).

(800) D. Anderle, M. Petrikova, and P. Kováč, *J. Chromatogr.*, **58**, 209 (1971).

(801) D. Anderle, P. Kováč, and H. Anderlová, *J. Chromatogr.*, **64**, 368 (1972).

(802) P. Kováč, *Chem. Zvesti*, **25**, 460 (1971).

(803) G. O. Aspinall and C. C. Whitehead, *Can. J. Chem.*, **48**, 3850 (1970).

chains of mesquite gum provided data on a large number of methyl ethers of L-arabinose that were separated either as their methyl glycosides or as their lactones. A paper on the pectic polysaccharides from the cotyledons of white mustard also contains data on many L-arabinose derivatives.^{781c}

2. Hexoses

In the galactose series, Ovodov and Pavlenko examined 10 ethers as their methyl glycosides on three columns, of which neopentyl glycol succinate was found to give the best resolution.⁸⁰⁴ There were, however, several cases of overlapping, and no mono-*O*-methylgalactosides nor the common 2,4-dimethyl ether were included in the study. Ovodov has also proposed the analysis of carbohydrate mixtures as the fully methylated alditols or methyl glycosides.²³¹ Poor resolution of arabinitol from xylitol, and glucitol from mannitol, as their methyl ethers, was obtained. The behavior of 2,3,4-, 2,3,6-, and 2,4,6-tri-*O*-methylgalactoses has been studied,⁸⁰⁵ and the separation of the methylated furanosides of galactose and galacturonic acid, together with the corresponding galactopyranosides, has been reported.⁸⁰⁶ The fully methylated furanosides, pyranosides, and septanosides of galactose have been separated by using a capillary column 25 meters long.⁸⁰⁷

Studies by Bayer and Widder⁸⁰⁸⁻⁸¹⁰ of the ring size of sugars, and of the existence of carbonyl forms, provided useful data on the fully methylated glycosides of arabinose, glucose, galactose, and fructose. Work by Anet⁸¹¹⁻⁸¹⁵ on the alkaline degradation of monosaccharides provided data on the methyl glycosides and other derivatives of 2,3,4,5-, 2,3,4,6-, and 2,3,5,6-tetra-*O*-methylglucose. In part, Anet⁸¹³ disagreed with certain results of Bayer and Widder.⁸⁰⁹

(804) Yu. S. Ovodov and A. F. Pavlenko, *J. Chromatogr.*, **36**, 531 (1968).

(805) T. Yamakawa, N. Kiso, S. Handa, A. Makita, and S. Yokoyama, *J. Biochem. (Tokyo)*, **52**, 226 (1962).

(806) H. G. Walker, Jr., and R. M. McReady, *Can. J. Chem.*, **41**, 3133 (1963).

(807) K. Heyns, D. Müller, R. Stute, and H. Paulsen, *Chem. Ber.*, **100**, 2664 (1967).

(808) E. Bayer and R. Widder, *Ann.*, **686**, 181 (1965).

(809) E. Bayer and R. Widder, *Ann.*, **686**, 197 (1965).

(810) E. Bayer and R. Widder, *Anal. Chem.*, **36**, 1452 (1964).

(811) E. F. L. J. Anet, *Carbohydr. Res.*, **8**, 164 (1968).

(812) E. F. L. J. Anet, *Carbohydr. Res.*, **2**, 448 (1966).

(813) E. F. L. J. Anet, *Carbohydr. Res.*, **3**, 251 (1966).

(814) E. F. L. J. Anet, *Carbohydr. Res.*, **7**, 453 (1968).

(815) E. F. L. J. Anet, *Carbohydr. Res.*, **1**, 348 (1966).

Gee and Walker reported the separation of such methylated glucose and fructose derivatives, mainly tetra- and tri-methyl ethers, as would arise from methylated sucrose derivatives.⁶⁸⁸ At the same time, they showed that fully methylated di- and tri-saccharides could be separated by gas chromatography. In a study on sucrose lactate, Percival and Young⁴⁷⁹ showed that the isomeric methyl 1,3,4-, 1,3,6-, and 1,4,6-tri-*O*-methyl-*D*-fructosides have similar retention times on 15% butanediol succinate, but are resolved on 10% polyphenyl ether or 10% ethylene glycol adipate.

The separation of the methyl ethers of glucose as their acetylated methyl glycosides and as their acetylated glucitols has been studied by H. G. Jones and J. K. N. Jones,^{816,817} and the behavior of a series of methylated methyl glucosides was reported by Heyns and coworkers;⁸¹⁸ the results were used in connection with a mass-spectrometric study.

The use of trimethylsilyl derivatives for the separation of glucose ethers has been examined by S. Haworth and coworkers as part of a study on cellulose.⁸¹⁹ They found that the retention times of the anomers differ according to the number of methyl substituents and their position. The recoveries ranged from 90–107% (with a mean of $100.0 \pm 4.8\%$) and the molar response-factors of all the compounds studied were essentially identical. In some instances, a marked lessening in retention time was noted for certain silylated compounds. Thus, the trimethylsilyl derivatives of 3-*O*-methyl-, 2,3-di-*O*-methyl-, and 2,3,6-tri-*O*-methyl- α -*D*-glucose have approximately the same retention time. This marked lessening of the retention time of trimethylsilyl derivatives of certain methyl ethers is particularly noticeable with mannose derivatives, and will be described further here and in Sections XIX,1 (see p. 25) and XX,1 (see p. 27). They concluded⁸¹⁹ that all of the methyl ethers they studied could be quantitatively analyzed by using two columns. Another study^{819a} of the methyl ethers of glucose showed that sharp peaks are obtained when Carbowax 20 M treated with 2-nitroterephthalic acid is used as the liquid phase.

The separation of those methyl ethers of *D*-glucose likely to be encountered in research on cellulose derivatives has also been studied.⁸²⁰

(816) H. G. Jones and J. K. N. Jones, *Can. J. Chem.*, **47**, 3269 (1969).

(817) H. G. Jones, J. K. N. Jones, and M. B. Perry, *Abstr. Papers Amer. Chem. Soc. Winter Meeting*, CARB 17 (1966).

(818) K. Heyns, K. R. Sperling, and H. F. Grützmacher, *Carbohydr. Res.*, **9**, 79 (1969).

(819) S. Haworth, J. G. Roberts, and B. F. Sagar, *Carbohydr. Res.*, **9**, 491 (1969).

(819a) W. J. Lewicki and J. R. Edwards, *Anal. Lett.*, **3**, 151 (1970).

(820) S. Patel, J. Rivlin, T. Samuelson, O. A. Stamm, and H. Zollinger, *Helv. Chim. Acta*, **51**, 169 (1968).

The resolution of methyl ethers of D-glucitol as their acetates, trifluoroacetates, or trimethylsilyl derivatives was examined. For the last compounds, it was found that 2,6- and 3,6-dimethyl ethers are only partly separated, and that the 2- and 3-monomethyl ethers are not separated at all. This result accords with the observation that trimethylsilyl ethers of alditols are often poorly resolved.

In connection with g.l.c. studies, Kováč and coworkers described syntheses of 3,4- and 4,6-di-O-methyl-D-glucose,⁸²¹ 3,4,6-tri-O-methyl-D-glucose,⁸²² and methyl 6-O-methyl- and 2,6-di-O-methyl-D-glucofuranosides.⁸²³

Bhattacharjee and Gorin⁸²⁴ examined the separation of di-, tri-, and tetra-O-methylmannoses as their methyl glycosides, the trimethylsilyl ethers of the methyl glycosides, and the trimethylsilyl derivatives of the partially methylated sugars. The best system for separation was found to depend on the degree of methylation. For example, the pair of tetra-O-methyl isomers was not distinguishable as the methyl glycosides, but was separable as the trimethylsilyl derivatives of the sugars, whereas, for the di-O-methyl isomers, methyl glycosides or trimethylsilyl ethers of the glycosides were found preferable.

A methylation study by Handa and Montgomery⁷⁸² has been mentioned in Section XVI (see p. 10); they analyzed their mixtures as methyl glycosides or as the trimethylsilyl ethers of the glycosides. The partial methylation of methyl α -D-mannopyranoside has also been studied by Fournet and Montreuil.^{824a} They showed that a column of Carbowax 6000 resolved all possible di-, tri-, and tetra-methyl ethers except the 2,3,4- and 3,4,6-tri-O-methyl derivatives, which, however, are separable on the same column as their trimethylsilyl derivatives.

Syntheses and g.l.c. characteristics of 2,4- and 3,6-di-O-methyl-D-mannose⁸²⁵ and of 2,3,6- and 2,4,6-tri-O-methyl-D-mannose⁸²⁶ have been described.

The separation of 2,3,4,6-tetra-O-methyl-D-glucose, -D-mannose, and -D-galactose on one liquid phase is almost impossible when these compounds are used as their methyl glycosides or alditol acetates.^{734,793,826a} If, how-

(821) P. Kováč and Ž. Longauerová, *Chem. Zvesti*, **26**, 179 (1972).

(822) P. Kováč, *Chem. Zvesti*, **24**, 218 (1970).

(823) P. Kováč and M. Petriková, *Chem. Zvesti*, **26**, 71 (1972).

(824) S. S. Bhattacharjee and P. A. J. Gorin, *Can. J. Chem.*, **47**, 1207 (1969).

(824a) B. Fournet and J. Montreuil, *J. Chromatogr.*, **75**, 29 (1973).

(825) G. Alfredss, P. J. Garegg, and B. Lindberg, *Acta Chem. Scand.*, **24**, 2671 (1970).

(826) Y. M. Choy and A. M. Unrau, *Carbohydr. Res.*, **17**, 439 (1971).

(826a) E. D. M. Eades, D. H. Ball, and L. Long, Jr., *J. Org. Chem.*, **31**, 1159 (1966).

ever, the acetates of the free sugars are employed, good resolution is obtained.^{826b} The same system may be used to resolve, readily, the 2,3,6-trimethyl ethers of D-glucose and D-mannose, which, as alditol acetates, have similar retention times.

Timell and coworkers⁷⁹⁷ found that isomeric methyl di-O-methyl-D-glucosides are well resolved as their peracetates.

3. Amino Sugars

Hakomori methylation of amino sugars yields the *N*-methyl derivatives, the g.l.c. characteristics of which have been described by Gorin and Magus.⁸²⁷ The synthesis of the needed derivatives of 2-amino-2-deoxy-D-glucose⁸²⁸ and -D-galactose⁸²⁹ has been described, and other g.l.c. data were given in papers on a yeast mannan⁸³⁰ and on blood-group substances.⁷¹⁷ Separation of the methyl ethers of 2-acetamido-2-deoxy-D-glucitol as their acetates has been described by Perry and Webb.^{830a}

4. Alditols and Aldononitriles

The problem of multiple peaks complicates the separation of methylated glycosides as much as in the separation of the parent monosaccharides. An important advance in the analysis of methylated sugars was the demonstration by Lindberg and coworkers⁸³¹ that many compounds may be successfully separated as their alditol acetates (which, necessarily, only give a single peak). A further advantage of these derivatives is that they lend themselves to mass-spectroscopic identification (see Section XXV; p. 37). One disadvantage of such compounds is that an ambiguity is introduced when the derived alditol is symmetrical; this may be overcome by using sodium borodeuteride for the reduction, or, alternatively, methylated sugars may be converted into their aldononitrile acetates.^{394a,799} The publications of Lindberg and coworkers^{734,831} (see also, Table XXVI, p. 89) contain data on a wide range of sugars. The differentiation of tri-O-methyl-D-glucitols has been studied by Nikaido⁸³²

(826b) G. M. Beaulieu, G. G. S. Dutton, and R. H. Walker, *Carbohydr. Res.*, **23**, 430 (1972).

(827) P. A. J. Gorin and R. J. Magus, *Can. J. Chem.*, **49**, 2583 (1971).

(828) P. A. J. Gorin and A. J. Finlayson, *Carbohydr. Res.*, **18**, 269 (1971).

(829) P. A. J. Gorin, *Carbohydr. Res.*, **18**, 281 (1971).

(830) P. A. J. Gorin, *Can. J. Chem.*, **49**, 527 (1971).

(830a) M. B. Perry and A. C. Webb, *Can. J. Chem.*, **47**, 4091 (1969).

(831) H. Björndal, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, **21**, 1801 (1967).

(832) H. Nikaido, *Eur. J. Biochem.*, **15**, 57 (1970).

in connection with the lipopolysaccharide of *Salmonella typhimurium*, and 2- and 4-*O*-methylabequose have been synthesized.⁸³³

Stephen and coworkers have made a detailed study of the separation⁸³⁴ of methylated galactitol acetates, and of their identification by nuclear magnetic resonance spectroscopy;⁸³⁵⁻⁸³⁷ the separation of methylated xylitol acetates⁷⁹⁹ was mentioned earlier in this Section (see p. 18).

A check list of model studies is provided in Table XV (see p. 54).

XVIII. METHYLATED GLYCOSIDES

The first application of g.l.c. in the carbohydrate field was to the methyl glycosides of methylated monosaccharides.¹ Such compounds are readily available by methanolysis of a methylated polysaccharide, and identification of methylated sugars has long been a classic method of investigating polysaccharide structures (see Section XVI; p. 10). These methylated glycosides may be obtained from methylated polysaccharides, either the original polysaccharide, a carboxyl-reduced material, or a degraded polysaccharide formed by partial hydrolysis, enzymolysis, or periodate oxidation; or, they may be obtained from neutral or acidic oligosaccharides that have been derived from polysaccharides.

The article by Bishop⁴ detailed the early work in this field, and gave Tables of the relative retention times of the methylated sugars commonly isolated in structural work on polysaccharides; further data were given by H. G. Jones.⁷³² The method has since been used in studies on a wide variety of polysaccharides, but, although improved instrumentation and better column-packings have facilitated certain aspects, no new principles have been elaborated.

Separation of methylated sugars as their methyl glycosides suffers from the disadvantage, discussed in Part I in Sections IV (Vol. 28, p. 38) and VI (Vol. 28, p. 51), that multiple peaks are usually obtained from each component. As with the unsubstituted monosaccharides, the profile of the individual peaks, together with their retention times,

(833) K. Stellner, O. Westphal, and H. Mayer, *Ann.*, **738**, 179 (1970).

(834) G. R. Woolard, A. M. Stephen, and E. B. Rathbone, *S. Afr. Med. J.*, **42**, 793 (1968).

(835) E. B. Rathbone, A. M. Stephen, and G. R. Woolard, *S. Afr. Med. J.*, **42**, 793 (1968).

(836) E. B. Rathbone, A. M. Stephen, and K. G. R. Pachler, *Carbohydr. Res.*, **20**, 141 (1971).

(837) E. B. Rathbone, A. M. Stephen, and K. G. R. Pachler, *Carbohydr. Res.*, **20**, 357 (1971).

may, however, serve for characterization because of the wealth of data that has now been accumulated for methyl glycosides.^{4,732} In certain instances, one of the anomers of a glycoside may be obtained readily in a crystalline state, thus making identification more positive (for example, methyl 2,3,4-tri-*O*-methyl- β -D-xylopyranoside).

Methyl glycosides are of their greatest use when only a few methylated sugars are anticipated, as with polysaccharides of simple structure and, in particular, with oligosaccharides. They are useful where identification must be based on peak profile and retention times, but, when characterization is to be achieved by mass spectrometry, other derivatives, such as alditol acetates, are preferable (see Section XXI,2; p. 30). Glycosides have the advantage over alditols that they may be hydrolyzed to the free sugar for further characterization.

A polar, ester-type of column (for example, butanediol succinate) is commonly used for the separation of methyl glycosides, with polyphenyl ether as a nonpolar alternative. In view of the very many separations that have been effected by using methyl glycosides, diverse ranges of conditions and columns have been developed; these are detailed in Table XVI (see p. 56).

Sometimes, a simple change of column is unsuccessful in ensuring resolution of certain mixtures of glycosides, or a glycoside may lack sufficient volatility. In such circumstances, the glycoside may be converted into a derivative, usually a trimethylsilyl ether or acetate, or the sugar may be transformed into some other type of compound. These alternatives are examined in Sections XIX to XXII (see pp. 24-33).

Table XVI (see p. 56) lists those polysaccharides whose structures have been examined by using the methylation technique and the methylated sugars have been separated as their methyl glycosides. In a few studies, g.l.c. has been used only to check on the identity of fractions separated by column chromatography. For convenience, Table XVI is subdivided according to the type of polysaccharide involved. In a similar way, Tables XVII (see p. 72), XVIII (see p. 76), and XIX (see p. 79) list those neutral, acidic, and basic oligosaccharides whose structures have been investigated in like manner by using g.l.c. Typical retention-times have been tabulated.⁷³²

XIX. DERIVATIVES OF METHYLATED GLYCOSIDES

Methyl glycosides having a low degree of substitution are often insufficiently volatile to be suitable for g.l.c., and isomeric glycosides may have similar retention times. In order to lessen or change retention times, the methylated methyl glycosides may themselves be converted

into such derivatives as the per(trimethylsilyl) ether or the peracetate; the former type is the more volatile.

1. Trimethylsilyl Ethers

Bauer and coworkers³⁷⁹ were the first to investigate the separation of methylated methyl glycosides as their trimethylsilyl ethers, but it was Ballou and his group^{175,794a,838,839} who demonstrated its utility in the mannose series, where the methyl glycosides of 2,4,6- and 3,4,6-tri-*O*-methylmannose cannot be separated by g.l.c. Conversion of these methyl glycosides into their trimethylsilyl derivatives permitted separation of the latter, but, more important, it greatly lessened their retention time (T_R). Thus, where methyl 2,3,4,6-tetra-*O*-methylmannoside had T_R 1.0, isomeric methyl tri-*O*-methylmannosides had T_R 2.3, but, when silylated, the latter were separable, with T_R 0.6 and 0.8. Similar results have been reported by Bishop and coworkers⁷⁹⁰ in studies on yeast mannans. The change in T_R for methyl di-*O*-methylmannosides is even more striking; from T_R 6.2 before to 0.33 after silylation.⁷⁹⁰

The presence of 2,3,4- and 2,4,6-tri-*O*-methyl-*D*-mannoses had been overlooked in earlier work⁸⁴⁰ in which the separation of the methyl mannosides had been attempted on Apiezon M. When the methyl glycosides were separated as their trimethylsilyl ethers on Carbowax 6000, resolution of the 2,3,4, 2,4,6, and 3,4,6 isomers was achieved. Bauer and coworkers^{841,842} have continued with the application of this method in their work on yeast mannans.

Bhattacharjee and Gorin⁸²⁴ utilized trimethylsilyl derivatives of methyl mannosides in a model study on mannose ethers, and recommended these compounds for the separation of the dimethyl isomers in particular. Handa and Montgomery⁷⁸² likewise used trimethylsilyl ethers in a study of the partial methylation of methyl α -*D*-mannopyranoside, and Choy and Unrau⁸²⁶ gave similar data for 2,3,6- and 2,4,6-tri-*O*-methyl-*D*-mannose.

Gorin and coworkers applied this type of separation in work on yeast galactomannans⁹⁷⁵ and mannans,⁸⁴³ as have Srivistava and coworkers⁸⁹² in examining a galactomannan from *Sesbania grandiflora*.

(838) T. S. Stewart and C. E. Ballou, *Biochemistry*, **7**, 1855 (1968).

(839) C. E. Ballou, *Accounts Chem. Res.*, **1**, 366 (1968).

(840) C. T. Bishop, F. Blank, and P. E. Gardner, *Can. J. Chem.*, **38**, 869 (1960).

(841) D. Šikl, L. Masler, and Š. Bauer, *Collect. Czech. Chem. Commun.*, **35**, 2965 (1970).

(842) L. Masler, D. Šikl, A. Kockova, and Š. Bauer, *J. Gen. Microbiol.*, **65**, 185 (1971).

(843) P. A. J. Gorin, J. F. T. Spencer, and S. S. Bhattacharjee, *Can. J. Chem.*, **47**, 1499 (1969).

In a study on the vinylation of methyl α -D-glucopyranoside, it was found that silylation of the O-vinylglucosides caused decomposition but, after reduction, the resulting ethyl ethers were successfully separated as the trimethylsilyl ethers of the methyl O-ethylglycosides.⁸⁴⁴

The problem of similar retention-times occurs not only with isomeric compounds but also in mixtures containing deoxy sugars. Thus, 2,3-di-O-methyl-L-rhamnose and 2,3,4,6-tetra-O-methyl-D-glucose are inseparable as their methyl glycosides, but, after trimethylsilylation of the mixture, resolution is readily achieved, the rhamnoside being eluted first.⁸⁴⁴ Larsson and Samuelson⁸⁴⁵ used the same principle in a study of the structure of an O-(α -D-galactopyranosyluronic acid)-L-rhamnopyranose 1,2':-1',2-dianhydride.

Zinbo and Timell⁸⁰¹ studied the separation of O-methylxyloses in the form of different derivatives, including the trimethylsilyl ethers of the methyl glycosides. Interestingly, although the separation of the less-volatile compounds was improved, there was no change in the relative order of elution after trimethylsilylation. This result is in marked contrast to that for compounds having the *manno* configuration. Timell and coworkers⁸⁴⁶⁻⁸⁵⁰ routinely used these derivatives in studies on wood polysaccharides.

2. Acetates

Acetylation does not greatly increase volatility, and thus, there are few examples of separations involving acetates of methyl glycosides, although J. K. N. Jones and coworkers used such derivatives in their model studies on L-arabinose⁷⁹⁸ and D-glucose.⁸¹⁶

Rees and Samuel⁸⁸ separated methylated mannosides from gulosides (obtained from alginic acid) in this way, and Aspinall and Whitehead⁸⁵¹ similarly identified three methyl tri-O-methyl-D-galactopyranosides from mesquite gum. Timell and coworkers likewise resolved a mixture of galactosides from an acidic arabinogalactan,⁸⁵⁰ and have separated a mixture of methyl di-O-methyl-D-glucopyranosides, obtained by chromatography on silicic acid, by g.l.c. of the peracetates.^{797,847} Separation of

(844) G. M. Bebault and G. G. S. Dutton, *Can. J. Chem.*, **50**, 3373 (1972).

(845) K. Larsson and O. Samuelson, *Acta Chem. Scand.*, **26**, 837 (1972).

(846) M. J. Song and T. E. Timell, *Cell. Chem. Technol.*, **5**, 67 (1971).

(847) G. C. Hoffmann and T. E. Timell, *Svensk Papperstidn.*, **75**, 135 (1972).

(847a) G. C. Hoffmann and T. E. Timell, *Wood Sci. Technol.*, **4**, 159 (1970).

(848) G. C. Hoffmann and T. E. Timell, *Svensk Papperstidn.*, **75**, 241 (1972).

(849) G. C. Hoffmann and T. E. Timell, *Svensk Papperstidn.*, **75**, 297 (1972).

(850) Y.-L. Fu and T. E. Timell, *Svensk Papperstidn.*, **75**, 680 (1972).

(851) G. O. Aspinall and C. C. Whitehead, *Can. J. Chem.*, **48**, 3840 (1970).

the methyl 2,3-, 2,4-, 2,6-, and 4,6-di-*O*-methyl-*D*-glucopyranoside peracetates was accomplished in less than 30 minutes on a column of ethylene glycol succinate.⁸⁴⁷

Fink and Hay⁸⁵² used methyl glycoside acetates in a study on enzymic deacylation, and Scott and Hay⁸⁷³ used such a derivative to characterize 2,3,6-tri-*O*-methyl-*D*-glucose obtained from methylated, sugar-maple roots.

Tables XX (see p. 80) and XXI (see p. 82) record examples of separations that used trimethylsilyl ethers and acetates of methyl *O*-methylglycosides. Tables of retention times of trimethylsilyl ethers of methyl mannosides and of acetates of methyl glucosides are available.⁷³²

XX. METHYLATED SUGARS

Another method for the separation of methylated compounds consists in converting the reducing, methylated sugar into the trimethylsilyl derivative or, less commonly, into the acetate. Multiple peaks can be formed, but, although these may complicate the chromatogram, their relative intensities and positions may aid in the identification. Alternatively, methylated sugars may be converted into their lactones.

I. Trimethylsilyl Derivatives

Sephton⁸⁵² was the first to apply trimethylsilylation to the analysis of the products obtained from a methylated xylan, and he demonstrated the excellent separation obtained in this way between 2- and 3-*O*-methylxylose. This discovery was significant, as these two compounds, for example, are not separable as alditol acetates, and thus this method has been utilized in the study of other xylans.^{601,701,762}

Most of the examples of this technique are to be found in connection with separation of the methyl ethers of glucose and, in particular, its mono-*O*-methyl compounds. Saier and Ballou⁸⁵⁴ demonstrated the separation of glucose and its monomethyl ethers on a column of Carbowax, and Glaudemans and Fletcher⁸⁵³ were able to separate 2- and 3-*O*-methylglucose on SE-52. Similar separations were achieved by Norrman⁸⁵⁴ on a column of butanediol succinate (in a study on pustulan). The same author,⁸⁵⁵ working on dextran, noted that the peaks of the 4- and 6-ethers of the β -*D*-glucoside overlapped, and, with the dimethyl ethers, one peak for the 2,4-diether overlapped with that of the 3,4-diether. During a

(852) H. H. Sephton, *J. Org. Chem.*, **29**, 3415 (1964).

(853) C. P. J. Glaudemans and H. G. Fletcher, Jr., *Carbohydr. Res.*, **7**, 480 (1968).

(854) B. Norrman, *Acta Chem. Scand.*, **22**, 1623 (1968).

(855) B. Norrman, *Acta Chem. Scand.*, **22**, 1381 (1968).

study of a hepta-*O*-acetylmaltose, Hodge and coworkers⁴⁰² were able to separate all the mono-*O*-methylglucoses as their trimethylsilyl derivatives.

BeMiller and Wing²⁹⁶ found that the α -D-glucose peak overlapped that for 4-*O*-methyl- β -D-glucose, and Weill and Bratt⁸⁵⁶ observed a similar situation with D-glucose and its 6-methyl ether. Wing and BeMiller⁶⁵¹ also separated D-glucose and its 4-*O*-methyl-, -ethyl, and -butyl derivatives in this way, together with 4-deoxy-D-xylo-hexose. 3-*O*-Methyl-D-glucose was used as the standard in a kinetic study on the hydrolysis of pseudo-cellobiouronic acid, and the composition of the mixture was estimated by using trimethylsilyl derivatives.²⁹⁵ 3-*O*-Methyl-D-glucose has also been used in a mass-spectrometric study of trimethylsilyl derivatives.²⁰⁰

A model study has been performed on the separation of a variety of methylated glucoses,⁸¹⁹ and the results were applied to a structural investigation on partially methylated cotton cellulose.⁸⁵⁷ This model study used four column packings, three of which were deposited on glass beads. The ratio of the specific retention volumes of α and β anomers varied with the total number of methyl substituents and their positions. The molar responses of all the compounds examined were found to be the same (within experimental error), and quantitative analysis of mixtures of all glucose ethers methylated at O-2, O-3, or O-6, or any combination thereof, was possible with two columns. The authors⁸¹⁹ drew particular attention to the effect, on separations, of small changes in temperature, and demonstrated that an alteration from 123 to 125° greatly influences the separation of α -D-glucose, 3-*O*-methyl- β -D-glucose, and 2,6-di-*O*-methyl- β -D-glucose.⁸¹⁹ Arendt and Pacsu⁷⁹⁴ similarly used trimethylsilyl derivatives in work on the structure of cellulose.

In a model study⁸²⁴ on the methyl ethers of D-mannose, the 2,3,4,6- and 2,3,5,6-ethers, as well as the 2,5,6- and 3,5,6-tri-*O*-methyl-D-mannoses, were best distinguished as the trimethylsilyl derivatives of the free sugars, and Gray and Ballou⁷⁰⁰ used these derivatives in their examination of a mannan from *Mycobacterium phlei*.

The methylated galactoses obtained from tamarack galactan were analyzed as the per(trimethylsilyl) derivatives by Jiang and Timell,⁸⁵⁸ and the same derivatives were used by Larsson and Samuelson⁸⁴⁵ in a study of a galacturonic acid anhydride. 3-*O*-Methyl-D-galactose has been estimated in the hydrolyzate of a fungal heteropolysaccharide by using trimethylsilyl derivatives.³³³

Kováč and coworkers, in papers reporting the synthesis of 3,4- and

(856) C. E. Weill and M. Bratt, *Carbohydr. Res.*, **4**, 230 (1967).

(857) S. Haworth, D. M. Jones, J. G. Roberts, and B. F. Sagar, *Carbohydr. Res.*, **10**, 1 (1969).

(858) K.-S. Jiang and T. E. Timell, *Svensk Papperstidn.*, **75**, 592 (1972).

4,6-di-*O*-methyl-*D*-glucose⁸²¹ and 3,4,6-tri-*O*-methyl-*D*-glucose,⁸²² gave g.l.c. data on the trimethylsilyl derivatives of these compounds. Klemmer and coworkers⁸⁵⁹ gave similar data for the trimethylsilyl derivatives of 2,3,4,6-tetra-*O*-methyl-*D*-glucose and *D*-galactose in a paper concerned with the synthesis of trehalose and its analogs.

Gorin and Magus⁸²⁷ investigated the use of the trimethylsilyl derivatives of the methyl ethers of 2-deoxy-2-(methylamino)-*D*-glucose and the corresponding *D*-galactose compounds.

2. Acetates

The separation of methylated sugars as acetates appears to have first been reported by H. G. Jones, J. K. N. Jones, and Perry,⁸¹⁷ although the method was not mentioned in the ensuing publication.⁸¹⁶ The acetates are particularly useful for the separation of the 2,3,4,6-tetramethyl ethers of *D*-glucose, *D*-galactose, and *D*-mannose,^{826b} which, when all three are present together,⁷⁹³ are difficult to resolve by other means. The acetates of epimeric tri-*O*-methyl derivatives of *D*-glucose and *D*-mannose have very different retention-times; these derivatives also provide a convenient method for resolving mixtures of glucose and rhamnose ethers.⁸⁴⁴ Acetates of di-*O*-methylhexoses are sufficiently volatile to be used for g.l.c., but the full scope of this method has not yet been explored.^{826b} Both acetates and trimethylsilyl derivatives have the advantage that the free sugar may readily be regenerated for further characterization.

3. Lactones

The use of glycosides, trimethylsilyl derivatives, and acetates poses the usual problem of multiple peaks. Sugars are conveniently oxidized to aldonic acids, which lactonize spontaneously. Many lactones are themselves sufficiently volatile to be used directly for g.l.c., although, in practice, the method is commonly restricted to di-*O*-methylpentoses^{247,803,851} and more highly methylated sugars.²⁹⁹⁻³⁰¹ Sometimes, further characterization is afforded by obtaining the lactone in crystalline form⁶⁴⁰ or by transforming it into a crystalline derivative.⁶⁴¹

Tables XXII (see p. 83), XXIII (see p. 86), and XXIV (see p. 87) record investigations that utilized trimethylsilyl and acetyl derivatives or lactones. Retention times of trimethylsilyl derivatives of methylated mannoses are given elsewhere.⁷³²

(859) A. Klemmer, E. Buhe, R. Kutz, S. Chahin, and L. Kleefeldt, *Ann.*, **739**, 185 (1970).

XXI. ACYCLIC DERIVATIVES

The separation of methylated sugars by use of the derivatives discussed in Sections XVIII–XX (pp. 23–27) may in all cases, except lactones, be complicated by the formation of multiple peaks. Although the peak profile for a particular sugar can be used as a distinguishing feature, it is often more convenient to transform the sugar into a derivative that can give only a single peak. The use of lactones (see Section XX,3; p. 29) is one example of this approach, but reduction to the alditol is far more common.

Many of the comments in Part I, Sections III (see Vol. 28, p. 23) and VII (p. 56) are equally pertinent to partially methylated alditols, which may be separated as their trimethylsilyl ethers, acetates, or trifluoroacetates; of these, the acetates are most generally used.

1. Trimethylsilyl Ethers of Alditols

It was shown in Part I, Section VII (see Vol. 28, p. 57) that trimethylsilyl ethers of acyclic compounds lack the resolution of their cyclic counterparts, and the same holds true for the ethers of partially methylated alditols. Thus, in a model study⁸²⁰ concerned with the separation of those methylated sugars likely to be encountered in research on cellulose derivatives, the separation of *O*-methylglucitols as their trimethylsilyl ethers, as well as acetates and trifluoroacetates, was examined. It was found that the trimethylsilyl ethers of 2,6- and 3,6-di-*O*-methyl-*D*-glucitols were only partially separated, and the 2- and 3-*O*-methyl-*D*-glucitols were not resolved. Such ethers were, however, found to be satisfactory when applied to the methylated glucitols obtained in studies on a glucan extracted from a lichen⁸⁶⁰ and prepared from a flavonoid glycoside.^{781a} These ethers have also been used in separating such simple mixtures as 3-*O*-methyl-*D*-glucitol plus methyl glucosides.⁴⁰²

2. Alditol Acetates

With the advent of column packings capable of separating alditol acetates (see Part I, Section VII,2; p. 59), these derivatives were first utilized by Lindberg and coworkers,⁸³¹ who showed that the same liquid phase (ECNSS-M) is suitable for the separation of these compounds having all degrees of methylation. The simplicity of the chromatogram, coupled with the fact that the pattern of methylation in alditol acetates may readily be determined by mass spectrometry (see Section XXV;

(860) E. Hauan and O. Kjølberg, *Acta Chem. Scand.*, **25**, 2622 (1971).

p. 37), makes these compounds the preferred derivatives, where choice is possible. The wide range of polysaccharides whose structures have been examined by using this g.l.c. method is attested to by the entries in Table XXVI (see p. 89).

Lindberg and coworkers⁸³¹ recommended that retention times be related to those of two standards, one of which has a short and the other a long retention-time. Suitable compounds that are readily available are 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylgalactitol, with retention times of 0.48 and 6.35 with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol ($T_R = 1.00$). By their use, the relative retention-times of the components of an appropriate mixture can be found by interpolation.

Where the components of a mixture differ greatly in degree of methylation, temperature programming may be used with advantage, and may be started⁴³⁵ at as low as 50°. Even with the use of this technique, there are some compounds not readily separated as alditol acetates, and for which other derivatives are preferable; thus, 2- and 3-*O*-methylxyloses are not separable as the alditol acetates, but are resolved as the trimethylsilyl derivatives. With mixtures of unknown composition, it is thus prudent to investigate the separation by trying different derivatives.

Satisfactory separations of alditol acetates have been obtained for *O*-methyl-*O*-propylglucitols, where propylation was used because of the natural occurrence of an *O*-methylglucose,⁶⁵⁴ and for *O*-ethyl-*O*-methylglucitols prepared during an investigation into the lengths of the side chains in dextrans.⁷⁶⁵

Perry and Webb^{830a} studied the separation of the acetates of partially methylated 2-amino-2-deoxyglucitols, and such derivatives have been used in the examination of *Serratia marcescens*.^{860a} The related *N*-methyl compounds were encountered during work on *Salmonella* hybrids,⁷²⁸ and Gorin and Magus⁸²⁷ made a detailed study of the acetates of 2-deoxy-*O*-methyl-2-(methylamino) derivatives of *D*-glucitol and *D*-galactitol.

The great majority of separations of methylated alditol acetates has been performed on the liquid phase ECNSS-M, but certain compounds not separated on such a column may be resolved by using another liquid phase, namely, polyphenyl ether (OS-138) (for example, 2,3-di-*O*-methylrhamnitol and 2,3,4,6-tetra-*O*-methylglucitol^{831,844}) or butanediol succinate (for example, 2,4,6- and 3,4,6-tri-*O*-methylmannoses⁸⁶¹⁻⁸⁶³).

(860a) G. A. Adams and P. P. Singh, *Biochim. Biophys. Acta*, **187**, 457 (1969).

(861) G. G. S. Dutton and Y. M. Choy, *Carbohydr. Res.*, **21**, 169 (1972).

(862) Y. M. Choy and G. G. S. Dutton, *Can. J. Chem.*, **51**, 198 (1973).

(863) Y. M. Choy, G. G. S. Dutton, K. B. Gibney, S. Kabir, and J. N. C. Whyte, *J. Chromatogr.*, **72**, 13 (1972).

Uronic acids may be identified and determined by reduction to the alditols and conversion thereof into alditol acetates. An acid commonly encountered in glycuronans is 4-*O*-methyl-*D*-glucuronic acid, but, on an ECNSS-M column, 4-*O*-methylglucitol and galactitol acetates are not well separated; however, they are cleanly resolved on butanediol succinate.^{467,863} In some instances, the degree of separation achieved with ECNSS-M may be markedly influenced by the nature of the solid support used. Thus, this liquid phase on Chromosorb not acid-washed may give longer retention-times, but improved resolution.^{417,793,863}

Another instance where the choice of column is critical is with 6-*O*-methyl-*D*-galactitol (1-*O*-methyl-*L*-galactitol) pentaacetate and β -*D*-galactopyranose pentaacetate, which are not separated on a column of 20% of Apiezon M, but are resolved by a liquid phase containing 5% of Apiezon M and 1% of poly(ethylene glycol).⁸⁶⁴

The liquid phase ECNSS-M has relatively low thermal stability, and it is therefore somewhat surprising that it has been so widely used when other, more stable, phases are available.⁸⁶³ Lönngren and Pilotti⁴¹⁹ commented unfavorably on ECNSS-M, and proposed use of a liquid phase of greater stability, having similar polarity and characteristics, namely, OV-225.

A three-component, liquid phase used by Albersheim and coworkers³⁷ for the separation of alditol acetates has also been found to give good resolution of partially methylated glucitols.^{865,866}

An alternative method for resolving difficultly separable combinations (for example, 2,3-di-*O*-methylrhamnitols and 2,3,4,6-tetra-*O*-methylglucitol acetates) is to use a "support-coated, open tube" (SCOT column) but these are commercially available for only a restricted number of instruments.^{435,437,439,440,445,713,781c}

3. Alditol Trifluoroacetates

The use of trifluoroacetates has been particularly recommended²²³ for the separation of mono-*O*-methylglucitols, these esters of which are readily resolved on a column of 1% of XE-60 used in conjunction with temperature programming. In work on cellulose derivatives, the separation of *O*-methylglucitols as their per(trifluoroacetates) was investigated, but it was found that, although satisfactory resolution could be obtained, quantitative relationships were not reproducible, and the ester of 2,3,4,6-tetra-*O*-methylglucitol is too volatile to be detected with the instrument

(864) M. Duckworth and J. R. Turvey, *Biochem. J.*, **113**, 687 (1969).

(865) J. N. C. Whyte, *Carbohydr. Res.*, **16**, 220 (1971).

(866) J. N. C. Whyte and J. R. Englar, *Can. J. Chem.*, **49**, 1302 (1971).

used.⁸²⁰ Trifluoroacetates have also been used in the examination of a flavonoid glycoside.^{781a}

4. Dithioacetals and Aldonitriles

Elimination of multiple peaks may also be achieved by conversion of a sugar into the dithioacetal or the nitrile. The suitability of the former type of derivative for g.l.c. appears to have been explored only in a model study on xylose methyl ethers.⁷⁹⁹ Lance and J. K. N. Jones⁷⁹⁹ also demonstrated that the acetylated nitriles derived from these ethers give sharp, single peaks, and permit 2- and 3-O-methyl-D-xylose to be distinguished from each other, a resolution not achieved by using alditol acetates. Nitriles have been used by Bouhours and Cheshire⁸⁶⁷ to demonstrate the occurrence of the same two xylose ethers in peat, and by Bacon and Cheshire⁸⁶⁸ to prove the natural existence of 3-O-methyl-D-galactose in leaves.

Reduction of some methylated sugars produces a symmetrical alditol; this can introduce ambiguities when the pattern of substitution is to be determined by mass spectrometry (see Section XXV; p. 37). Such problems are obviously avoided by the use of dithioacetals or nitriles. For this reason, Dmitriev and coworkers^{394a} investigated both the g.l.c. and mass spectrometry of acetylated nitriles.

Investigations that have involved separations of trimethylsilyl ethers of alditols are listed in Table XXV (see p. 88); Tables XXVI (see p. 89), XXVII (see p. 99), and XXVIII (see p. 100) record separations based on alditol acetates, trifluoroacetates, and aldonitrile acetates, respectively. The retention times of alditol acetates have been tabulated.^{732,734,831}

XXII. PERIODATE DEGRADATION OF METHYLATED SUGARS

Despite the great sensitivity of g.l.c., and its ability to resolve mixtures difficult or impossible to separate by other means, the technique is not a universal "panacea" and may sometimes fail. One of the more stringent situations exists where a polysaccharide contains several different types of linkage, and thus, after methylation, gives rise to a group of isomeric compounds. This problem is well illustrated by a series of studies on synthetic polysaccharides that have been shown to have random structures. For example, hydrolysis of a methylated, synthetic rhamnan gave a mixture of five isomeric di-O-methylrhamnoses that was

(867) J.-F. Bouhours and M. V. Cheshire, *Soil Biol. Biochem.*, **1**, 185 (1969).

(868) J. S. D. Bacon and M. V. Cheshire, *Biochem. J.*, **124**, 555 (1971).

not resolvable by the g.l.c. techniques existing at that time.⁶⁴¹ By sequential borohydride reduction, periodate oxidation, and a second reduction, the isomeric six-carbon sugars were converted into compounds, readily separable by g.l.c., containing three, four, five, or six carbon atoms. It is worth stressing that, in this instance, each di-*O*-methylrhamnose gives rise to a unique degradation product;⁶⁴¹ the latter derivative may be identified directly, or may be demethylated to the parent polyhydric compound, identification of which often suffices to indicate the positions of the methoxyl groups in the original compound.

This degradative technique has been applied in the study of gums, which commonly give rise to complex mixtures of methylated sugars.^{106,637,869,870} The method may also be used as part of a rigorous proof of the structure of a particular compound isolated, as in the conversion of a tri-*O*-methyl-*D*-mannose into a tri-*O*-methyl-*L*-arabinose,^{775,871,872} or of a synthetic product.⁸⁷³

Similar transformations are useful in determining the nature of heptoses (either before³⁵⁹ or after methylation),⁸⁷⁴ for demonstrating the existence of hexofuranose units in a polysaccharide,²⁰⁶ and for studying the products obtained from amino sugars by reaction with ninhydrin.^{880a}

It should be noted that, in most of the examples cited, where the products of periodate oxidation were separated by g.l.c., these products were sufficiently volatile to be used as such, without conversion into derivatives. In contrast, Rees and Samuel⁸⁸ commented on the difficulty of handling the acetates of di-*O*-methyltetritols because of their volatility.

Data on the periodate degradation of the methyl ethers of *L*-arabinose,⁸⁷⁵ *D*-galactose,²⁰⁷ and *D*-mannose⁶⁴⁰ may be found in papers reporting the structures of the corresponding synthetic glycans, and of *D*-glucose in a publication by Saier and Ballou.⁷⁸⁹

Representative examples of periodate oxidations are given in Table XXIX (see p. 101). In certain studies, the degradation products were not separated by g.l.c., or were not directly isolated, but they are included for completeness. Furthermore, some workers omitted the second borohydride reduction and isolated a reducing sugar, or they oxidized the original sugar or its glycoside (instead of the corresponding alditol), or both. These variations will be obvious on study of the individual articles.

(869) A. M. Unrau, *Can. J. Chem.*, **42**, 916 (1964).

(870) A. Misaki and S. Yukawa, *J. Biochem. (Tokyo)*, **59**, 511 (1966).

(871) G. O. Aspinall and T. B. Christensen, *J. Chem. Soc.*, 2673 (1965).

(872) F. Blank and M. B. Perry, *Can. J. Chem.*, **42**, 2862 (1964).

(873) I. R. Siddiqui and V. L. N. Murty, *Carbohydr. Res.*, **8**, 477 (1968).

(874) W. Droge, O. Lüderitz, and O. Westphal, *Eur. J. Biochem.*, **4**, 126 (1968).

(875) G. G. S. Dutton and A. M. Unrau, *Can. J. Chem.*, **43**, 924 (1965).

XXIII. MISCELLANEOUS ETHERS

The use of ethyl and propyl ethers in conjunction with, or instead of, methylation was discussed in Section XVI (see p. 10), because these derivatives are logically included with methyl ethers.

The methods of g.l.c. have also been extended to 2-hydroxyethyl,⁸⁷⁶ 2-aminoethyl,⁸⁷⁷ and similar ethers. Such compounds have usually been encountered in work concerned with the preparation of modified starch or cellulose. These types of compound have little direct bearing on the theme of the present article and will therefore not be discussed in detail, but representative applications are given in Table XXX (see p. 102).

XXIV. DETERMINATION OF OTHER GROUPS

Formaldehyde is produced by the periodate oxidation of certain carbohydrates, and, although there has as yet been no report of a gas-liquid chromatographic determination of formaldehyde obtained in this way, formaldehyde has been so determined in other contexts. Mixtures of formaldehyde and methanol have been analyzed,⁸⁷⁸ columns of acetylated polyesters⁸⁷⁹ or sucrose octaacetates⁸⁸⁰ being used. The system formaldehyde-water-methanol in which the concentration of formaldehyde was varied from 0.06 to 46.1% was examined by use of a column of Porapak N, and references to previous methods of determination were given.⁸⁸¹ K. Jones evaluated a wide range of supports and liquid phases for analyzing aqueous formaldehyde.⁸⁸²

In Enterobacteriaceae, certain of those polysaccharides which comprise the M antigens have been found to carry some acetal substituents, such as carboxyethylidene, ethylidene, isopropylidene, or methylidene.⁴⁴³ Such groupings may be identified by injecting an aqueous hydrolyzate onto columns of Carbowax⁴⁴⁴ or⁸⁸³ OV-1.

The 2-hydroxyethyl group in 2-hydroxyethylated starch has been determined by a pyrolysis-gas chromatography technique in which the peak for acetaldehyde is used as a measure of the degree of substitution

(876) C. E. Lott, Jr., and K. M. Brobst, *Anal. Chem.*, **38**, 1767 (1966).

(877) E. J. Roberts and S. P. Rowland, *Can. J. Chem.*, **47**, 1571 (1969).

(878) F. Buscaron and M. Paraira, *Inform. Quím. Anal.* (Madrid), **23**, 31 (1969).

(879) T. Iguchi and T. Takiuchi, *Bunseki Kagaku*, **17**, 1081 (1968).

(880) R. S. Mann and K. W. Hahn, *Anal. Chem.*, **39**, 1314 (1967).

(881) F. Onuška, J. Janák, Š. Duraš, and M. Krémárová, *J. Chromatogr.*, **40**, 209 (1969).

(882) K. Jones, *J. Gas Chromatogr.*, **5**, 432 (1967).

(883) P. J. Garegg, B. Lindberg, T. Omm, and T. Holme, *Acta Chem. Scand.*, **23**, 2194 (1969).

(d.s.).⁸⁸⁴ The d.s. in *O*-ethylcellulose was estimated similarly by measuring, on a Poropak column, the acetic acid produced on oxidation with chromium trioxide.^{884a} The same method was used to determine the substituent content of several cellulose esters and ethers.⁸⁸⁵

In some polysaccharides, a fraction of the hydroxyl groups may actually occur as acetates. The proportion is conveniently estimated by subjecting the polysaccharide to methanolysis, and determining the acetic acid as methyl acetate by g.l.c.^{439,440,444,445,713,730} Acetic acid has also been estimated directly by use of a column of Porapak Q^{885a} or Tween 80.^{885b} Polysaccharides are also known that contain formate⁸⁸⁶ and succinate⁸⁸⁷ groups, but these do not appear to have been determined by g.l.c. in this context, although such studies have been made for acids in biological systems.^{887a,887b} 3-Hydroxytridecanoic acid and 3-hydroxypentadecanoic acid have been found in the lipopolysaccharide of *Veillonella*, and have been characterized by g.l.c. and mass spectrometry.^{887c} *N*-Acetyl groups may be determined in a similar way,^{885b,888,888a} and a g.l.c. procedure has been devised for the determination of sulfate in sulfated glycosaminoglycans.⁸⁸⁹

Water has been determined in starch hydrolyzates by dissolving the sample in methyl sulfoxide and measuring the ratio of the peak heights for water and sulfoxide by using⁸⁹⁰ a column of Porapak Q. Other applications include the quantitative determination of 5-(hydroxymethyl)-2-furaldehyde, which is a carbohydrate degradation-product,⁸⁹¹ the analysis of the thermal degradation-products of carbohydrates has been re-

- (884) H. Tai, R. M. Powers, and T. F. Protzman, *Anal. Chem.*, **36**, 108 (1964).
- (884a) H. Jacin and J. M. Slanski, *Anal. Chem.*, **42**, 801 (1970).
- (885) W. D. King and D. J. Stanonis, *Tappi*, **52**, 465 (1969).
- (885a) Y. Nishikawa, M. Tanaka, S. Shibata, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **18**, 1431 (1970).
- (885b) A. R. Archibald, J. Baddiley, and D. Button, *Biochem. J.*, **110**, 543 (1968).
- (886) I. W. Sutherland, *J. Chromatogr.*, **59**, 476 (1971).
- (887) T. Harada, *Arch. Biochem. Biophys.*, **112**, 65 (1965).
- (887a) M. G. Horning, E. A. Boucher, A. M. Moss, and E. C. Horning, *Anal. Lett.*, **1**, 713 (1968).
- (887b) T. Murai, A. Kawaguchi, H. Nakano, and H. Katsuki, *Agr. Biol. Chem.* (Tokyo), **35**, 242 (1971).
- (887c) D. G. Bishop, M. J. Hewett, and K. W. Knox, *Biochim. Biophys. Acta*, **231**, 274 (1971).
- (888) G. R. Shepherd and B. J. Noland, *Anal. Biochem.*, **26**, 325 (1968).
- (888a) B. Radhakrishnamurthy, E. R. Dalferes, and G. S. Berenson, *Anal. Biochem.*, **26**, 61 (1968).
- (889) S. R. Srinivasan, B. Radhakrishnamurthy, E. R. Dalferes, and G. S. Berenson, *Anal. Biochem.*, **35**, 398 (1970).
- (890) D. W. Vomhof and J. H. Thomas, *Abstr. Papers Amer. Chem. Soc. Meeting*, **157**, CARR 24 (1969).
- (891) H. Jacin, J. M. Slanski, and R. J. Moshy, *J. Chromatogr.*, **36**, 359 (1968).

viewed.⁸⁹² Aldonic acids have been proposed for the resolution of racemic alcohols.⁸⁹³

XXV. MASS SPECTROMETRY

The Introduction to this article (see p. 10) drew attention to the advances in the determination of polysaccharide structures that have accrued as a result of the combination of three techniques. Two of these, namely, methylation, and g.l.c. separations of the methylated sugars thus obtained, have been treated in previous Sections. It remains, therefore, to indicate the impact of mass spectrometry (m.s.) on certain aspects of the chemistry of polysaccharides.

Much of the earlier work on the mass spectrometry of carbohydrates was discussed in this Series by Kochetkov and Chizhov,⁸⁹⁴ and the same authors have presented certain practical aspects of the technique.⁸⁹⁵ Hanessian⁸⁹⁶ described the application of mass spectrometry to natural products containing sugars, and other reviews have been written by Rosenthal⁸⁹⁷ and Heyns and coworkers.⁸⁹⁸ Lindberg and coworkers⁷³⁴ discussed the combined use of gas-liquid chromatography and mass spectrometry (g.l.c.-m.s.) with reference to alditol acetates and, elsewhere in this Series (see Vol. 29, Chapter 3), Lönngren and Svensson⁷³³ amplified this topic. The purpose of the present Section is to collate those literature reports relevant to the determination of polysaccharide structures, but not to discuss such matters as the mechanism by which the fragments are formed, information on which can be found in the reviews and articles mentioned.

1. Trimethylsilyl Derivatives

Petersson and Samuelson^{899,900} showed that the fragmentation patterns of per(trimethylsilyl)ated and permethylated monosaccharides are similar, and demonstrated that mixed derivatives show analogous behavior. The mass spectra of trimethylsilyl derivatives of methylated sugars thus

(892) I. S. Fagerson, *Agr. Food Chem.*, **17**, 747 (1969).

(893) G. E. Pollock and D. A. Jermany, *J. Gas Chromatogr.*, **6**, 412 (1968).

(894) N. K. Kochetkov and O. S. Chizhov, *Advan. Carbohydr. Chem.*, **21**, 39 (1966).

(895) N. K. Kochetkov and O. S. Chizhov, *Methods Carbohydr. Chem.*, **6**, 540 (1972).

(896) S. Hanessian, *Methods Biochem. Anal.*, **19**, 105 (1971).

(897) A. Rosenthal, *Carbohydr. Res.*, **8**, 61 (1968).

(898) K. Heyns, H. F. Grützmacher, H. Scharmann, and D. Müller, *Fortschr. Chem. Forsch.*, **5**, 448 (1966).

(899) G. Petersson and O. Samuelson, *Svensk Papperstidn.*, **71**, 77 (1968).

(900) G. Petersson and O. Samuelson, *Svensk Papperstidn.*, **71**, 731 (1968).

permit the number and position of methyl groups in aldopentoses⁸⁹⁹ and aldohexoses⁹⁰⁰ to be assigned; substitution of a methyl group for a trimethylsilyl group gives a characteristic shift of the m/e value, corresponding to a mass difference of 58. The method is of general applicability, but has not been widely adopted for methylated sugars, as alditol acetates give simpler chromatograms and are thus the preferred derivatives. The method is, however, invaluable where mixtures are not separable as alditol acetates, as with 2- and 3-*O*-methylxyloses, and the structure of everninose, a naturally occurring, partially methylated disaccharide, has been investigated by using mass spectrometry of the trimethylsilyl derivative.^{900a}

Extensive use has been made of trimethylsilyl derivatives of aldono-lactones and related acids;^{499,500} such compounds were used to identify a new uronic acid, 4-*O*-methyl-L-iduronic acid, in pulp hydrolyzates,⁵²² and to examine an anhydride formed from an aldobiouronic acid.⁸⁴⁵ Samuelson and coworkers have similarly identified (*a*) products formed by isomerization of D-galacturonic acid,²⁶¹ (*b*) products of the reaction of D-glucose with ethylene oxide,⁹⁰¹ (*c*) aldonic acids in polysulfide pulps,⁵⁰² (*d*) hydroxy acids formed by treatment of hydrocellulose with alkali,⁵⁰⁴ and (*e*) glucopyranosylglycolic acids.⁵⁰⁸ Ethyl β -D-glucosiduronic acid, formed in animals as a metabolite of ethanol, has been characterized in like manner.⁵³⁵ It has been reported⁹⁰² that fluoroalcohol esters of carboxylic acids give clean spectra because the fragmentation pattern is spread over a wider range than for the nonfluorinated analogs. This property has not yet been applied in the carbohydrate field, but it may be useful for aldonic or uronic acids.

The fragmentation of the trimethylsilyl derivatives of 2-amino-2-deoxy-D-glucose and -D-galactose has been studied by Kärkkäinen and Vihko,¹⁴⁴ and of the latter compounds, by Heyns and coworkers.⁹⁰³ The German workers also used m.s. to examine glucosides and galactosides,⁸¹⁸ as well as the products obtained on pyrolysis of mono- and poly-saccharides.⁹⁰⁴

The ring size of ketoses^{652;905} and the structure of gangliosides^{154,356,905a} have been examined by using g.l.c.-m.s., and the mass spectra of per-*O*-(trimethylsilyl)alditols have been reported.⁹⁰⁶

(900a) A. K. Ganguly, O. Z. Sarre, and J. Morton, *J. Chem. Soc. (D)*, 1488 (1969).

(901) O. Ramnäs and O. Samuelson, *Carbohydr. Res.*, **6**, 355 (1968).

(902) R. M. Teeter, *Anal. Chem.*, **39**, 1742 (1967).

(903) K. Heyns, G. Kiessling, and D. Müller, *Carbohydr. Res.*, **4**, 452 (1967).

(904) K. Heyns and M. Klier, *Carbohydr. Res.*, **6**, 436 (1968).

(905) S. Karady and S. H. Pines, *Tetrahedron*, **26**, 4527 (1970).

(905a) G. Dawson and C. C. Sweeley, *J. Lipid Res.*, **12**, 56 (1971).

(906) G. Petersson, *Tetrahedron*, **25**, 4437 (1969).

The use of trideuteriomethylsilyl derivatives has been investigated by Waller and coworkers;¹⁴³ it is claimed that a comparison of the mass spectra of the labelled and unlabelled derivatives avoids the necessity for high-resolution instrumentation.⁹⁰⁷ Sweeley and coworkers²⁰⁰ published a detailed paper on the mass spectra of monosaccharides, glycosides, and amino derivatives, and used the data to interpret peaks caused by minor compounds formed on silylation. Similar techniques may be used for the determination of unresolved components.²⁰²

Although it has been generally accepted that diastereoisomers give virtually identical mass-spectra, Vink and coworkers⁹⁰⁸ have shown, with the aid of a computer, that peak intensities exhibit minor, but definite, differences that depend on the configuration of the sugar. Havlicek and colleagues^{908a} also commented on the influence of stereochemistry on the m.s. of pyranose derivatives.

Kochetkov, Chizhov, and their colleagues have demonstrated how m.s. may be used for linkage analysis of di-⁹⁰⁹ and tri-^{910,911} saccharides as their per(trimethylsilyl) derivatives. They have extended the method to disaccharide alditols,^{911a} which have also been studied by Kärkkäinen.³⁸⁹ Kamerling and coworkers^{912,912a} amplified the selection of disaccharides, and studied the mass spectra of 18 compounds representing all possible linkages from (1→1) to (1→6). Two groups have investigated the mass spectra of oligosaccharides containing fructose,^{913,914} and amino sugars

- (907) J. A. McCloskey, R. N. Stillwell, and A. M. Lawson, *Anal. Chem.*, **40**, 233 (1968).
- (908) J. Vink, J. H. W. Bruins Slot, J. J. de Ridder, J. P. Kamerling, and J. F. G. Vliegthart, *J. Amer. Chem. Soc.*, **94**, 2542 (1972).
- (908a) S. C. Havlicek, M. R. Brennan, and P. J. Scheuer, *Org. Mass Spectrom.*, **5**, 1273 (1971).
- (909) O. S. Chizhov, N. V. Molodtsov, and N. K. Kochetkov, *Carbohydr. Res.*, **4**, 273 (1967).
- (910) N. K. Kochetkov, O. S. Chizhov, and N. V. Molodtsov, *Tetrahedron*, **24**, 5587 (1968).
- (911) O. S. Chizhov, N. N. Malyshev, V. I. Kadentse, and G. F. Fridlyan, *Izv. Akad. Nauk SSSR*, 196 (1971).
- (911a) O. S. Chizhov, N. N. Malyshev, V. I. Kadentse, and N. K. Kochetkov, *Dokl. Akad. Nauk SSSR*, **194**, 836 (1970).
- (912) J. P. Kamerling, J. F. G. Vliegthart, J. Vink, and J. J. de Ridder, *Tetrahedron*, **27**, 4275 (1971).
- (912a) J. Vink, J. J. de Ridder, J. P. Kamerling, and J. F. G. Vliegthart, *Biochem. Biophys. Res. Commun.*, **42**, 1050 (1971).
- (913) W. W. Binkley, R. C. Dougherty, D. Horton, and J. D. Wander, *Carbohydr. Res.*, **17**, 127 (1971).
- (914) J. P. Kamerling, J. F. G. Vliegthart, J. Vink, and J. J. de Ridder, *Tetrahedron Lett.*, 2367 (1971); *Tetrahedron*, **28**, 4375 (1972).

have also been studied.⁹¹⁵ Incidental to these studies, Kamerling and co-workers have shown^{916,916a} that per(trimethylsilyl) derivatives of disaccharides give clear, proton magnetic resonance spectra from which the configuration of the glycosidic linkage may be determined. Similar observations have been made by Lindberg and coworkers.⁴⁸⁶ The structure of oligosaccharides obtained by Smith degradation may also be determined⁷⁸⁷ by g.l.c.-m.s. Trimethylsilyl derivatives give very weak peaks for the parent ion (M) or M - 15, and Karliner⁹¹⁷ suggested that 3-methyl-1-naphthyl glycosides of disaccharides are more suitable than trimethylsilyl derivatives. For the same reason, Johnson and coworkers^{918,918a,918b} proposed the use of 1-phenylflavazole derivatives.

Some aspects of the mass spectrometry of trimethylsilyl derivatives have been reviewed by de Wilt and Tsuchiya.^{918c}

2. Acetates

The use of alditol acetates for the separation of mixtures of partially methylated sugars not only simplifies the g.l.c. but also the mass spectra, because such compounds contain carbon to carbon bonds of only three types: those between (a) carbon atoms that each carries a methoxyl group, or (b) each bears an acetoxyl group, or (c) adjacent carbon atoms having one substituent of each type. Mass spectrometry of partially methylated alditol acetates thus gives rise to relatively few primary fragments that are characteristic of the pattern of substitution. This technique was introduced to carbohydrate chemistry by Lindberg and his associates,^{734,919} and has been employed extensively by them in subsequent structural studies, some of which are discussed next; others are listed in Table XXXI (see p. 103).

Lindberg and coworkers⁹¹⁹ showed that the fragmentation pattern of

- (915) J. P. Kamerling, J. F. G. Vliegenthart, J. Vink, and J. J. de Ridder, *Tetrahedron*, **27**, 4749 (1971).
- (916) J. P. Kamerling, M. J. A. de Bie, and J. F. G. Vliegenthart, *Tetrahedron*, **28**, 3037 (1972).
- (916a) J. P. Kamerling, D. Rosenberg, and J. F. G. Vliegenthart, *Biochem. Biophys. Res. Commun.*, **38**, 794 (1970).
- (917) J. Karliner, *Tetrahedron Lett.*, 3545 (1968).
- (918) G. S. Johnson, W. S. Ruliffson, and R. G. Cooks, *J. Chem. Soc. (D)*, 587 (1970).
- (918a) G. S. Johnson, W. S. Ruliffson, and R. G. Cooks, *Carbohydr. Res.*, **18**, 233 (1971).
- (918b) G. S. Johnson, W. S. Ruliffson, and R. G. Cooks, *Carbohydr. Res.*, **18**, 243 (1971).
- (918c) H. G. J. de Wilt and T. Tsuchiya, *Shitsuryo Bunseki*, **18**, 1294 (1970); *Chem. Abstr.*, **74**, 88,254 (1971).
- (919) H. Björndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967).

acetylated, methylated alditols is dependent only on the pattern of substitution of the alditol, and is independent of the configuration; this behavior has the advantage of minimizing the number of standards required. The source of many of the primary fragments was discussed in the original publication,⁹¹⁹ and it has subsequently been studied by using deuterium labelling.^{920,921}

The alditols obtained from a 2,3- and a 3,4-di-*O*-methylpentose will give the same mass spectrum, and thus an ambiguity is introduced by reducing the sugar. This difficulty may be overcome by using sodium borodeuteride as the reducing agent; the procedure is illustrated by a study on a fungal fucoxylomannan that gave 3,4-di-*O*-methyl-*D*-xylose.⁹²² Reduction with deuterides may be an advantage in other situations. Thus, the *D*-glucose arising from reduction of *D*-glucuronic acid may be differentiated from that originally present,^{484a,781b} and the reducing unit of an oligosaccharide may be distinguished from the others when the hydrolyzate is to be analyzed as alditol acetates.⁷¹² Mass spectrometry is likewise the most convenient way to determine the pattern of substitution in alditols possessing both ethyl and methyl groups,⁷⁶⁵ or both methyl and deuteriomethyl groups.⁷¹³

The fragmentation patterns described by the original authors^{734,919} are highly reproducible, and comparisons with standard spectra, although desirable, are not always necessary. Attention has been drawn⁸⁶³ to the fact that, sometimes, the presence of a peak having an intensity of less than 10 percent of the base peak⁷³⁴ may be the most significant in making an assignment. The same group of workers⁸⁶¹⁻⁸⁶³ demonstrated that the configuration of the methylated alditol, a question unresolved by mass spectrometry, may be determined by demethylation, and preparation of the alditol peracetate; these acetates may be purified by g.l.c. and, for *D*-glucitol, *D*-mannitol, and galactitol, are crystalline. Alditol acetates and their partially methylated derivatives give characteristic, circular dichroism spectra from which, by reference to standards, the configuration of each component in a polysaccharide may be determined.⁹²³

Further examples of the use of g.l.c.-m.s. for alditol acetates are given in Table XXXI (see p. 103); it is surprising how few research groups, apart from those originating this and the related method using trimethyl-

- (920) H. Björndal, B. Lindberg, A. Pilotti, and S. Svensson, *Carbohydr. Res.*, **15**, 339 (1970).
- (921) H. B. Boren, P. J. Garegg, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, **25**, 3299 (1971).
- (922) K. Axelsson, H. Björndal, S. Svensson, and S. Hammaström, *Acta Chem. Scand.*, **25**, 3645 (1971).
- (923) G. M. Bebault, J. M. Berry, Y. M. Choy, G. G. S. Dutton, N. Funnell, L. D. Hayward, and A. M. Stephen, *Can. J. Chem.*, **51**, 324 (1973).

silyl derivatives, have availed themselves of this technique—so admirably suited to the identification of milligram quantities of material. This situation may, in part, be attributable to the fact that the original investigators^{784,899} used a combined g.l.c.-m.s. instrument, but, even when the two instruments are physically separate,⁸⁶³ excellent results may be obtained except for very volatile compounds.

3. Methyl Derivatives

Much of our knowledge on the fragmentation patterns of carbohydrate derivatives arises from studies on methylated monosaccharides,⁸⁹⁴ but in structural investigations, such compounds are seldom subjected to mass spectrometry.⁹²⁴ On the other hand, the molecular weight of a permethylated oligosaccharide is significantly less than that of the per(trimethylsilyl) derivative. For this reason, the mass spectra of disaccharides as their permethylated alditols have been determined by Chizhov and coworkers,^{911a} Kärkkäinen,⁹²⁵ and Krone and Beckey.⁹²⁶ Trisaccharides have also been studied by Kärkkäinen, either as permethylated glycosides⁹²⁷ or as alditols.⁹²⁸

In a series of papers, Kováčik and Bauer⁹²⁹⁻⁹³² described their studies of the fragmentation patterns of uronic acids and acidic oligosaccharides; Kováčik and Kováč⁹³³ examined methyl *O*-methyl-D-xylofuranosides, compounds sufficiently volatile to be subjected to mass spectrometry without prior formation of volatile derivatives.

4. Trifluoroacetates

Trifluoroacetates have been little used as derivatives for g.l.c. (see Part I, Section III,3; Vol. 28, p. 36), and mass spectra have thus far been recorded only for trifluoroacetylated alditols⁹³⁴ and nucleosides.⁹³⁵

- (924) N. K. Kochetkov, N. S. Wulfson, O. S. Chizhov, and B. M. Zolotarev, *Tetrahedron*, **19**, 2209 (1963).
- (925) J. Kärkkäinen, *Carbohydr. Res.*, **14**, 27 (1970).
- (926) H. Krone and H. D. Beckey, *Org. Mass Spectrom.*, **5**, 983 (1971).
- (927) J. Kärkkäinen, *Carbohydr. Res.*, **17**, 1 (1971).
- (928) J. Kärkkäinen, *Carbohydr. Res.*, **17**, 11 (1971).
- (929) V. Kováčik and Š. Bauer, *Collect. Czech. Chem. Commun.*, **34**, 326 (1969).
- (930) V. Kováčik, Š. Bauer, and P. Šipoš, *Collect. Czech. Chem. Commun.*, **34**, 2409 (1969).
- (931) V. Kováčik and Š. Bauer, *Carbohydr. Res.*, **8**, 282 (1968).
- (932) V. Kováčik, Š. Bauer, and J. Rosík, *Carbohydr. Res.*, **8**, 291 (1968).
- (933) V. Kováčik and P. Kováč, *Carbohydr. Res.*, **24**, 23 (1972).
- (934) O. S. Chizhov, B. A. Dmitriev, B. M. Zolotarev, A. Ya. Chernyak, and N. K. Kochetkov, *Org. Mass Spectrom.*, **2**, 947 (1969).
- (935) W. A. Koenig, L. C. Smith, P. F. Crain, and J. A. McCloskey, *Biochemistry*, **10**, 3968 (1971).

The former compounds give simple fragmentation patterns having intense peaks in the high mass range. These derivatives are particularly useful for the detection and location of a deoxy group in a molecule. In view of their volatility, trifluoroacetates may prove useful for oligosaccharide sequencing (see Part I, Section VIII,3; Vol. 28, p. 70).

5. Aldononitrile Acetates

Aldononitriles give sharp, single peaks on g.l.c., and their methylated derivatives give mass spectra characteristic of the pattern of substitution. Their use instead of alditol acetates has been recommended,^{394a} as the possible ambiguity introduced on reduction of the sugar is avoided.

6. Other Derivatives

In order to obtain strong, molecular-ion peaks, the use of *N*-phenylglycosylamines ("anilides")⁸⁶⁵ or phenylosazones⁹³⁶ has been recommended, in addition to the 3-methyl-1-naphthyl⁹¹⁷ and 1-phenylflavazole⁹¹⁸ derivatives mentioned in Section XXV,1 (see p. 37). Diols are possible products of the periodate degradation of polysaccharides, and the mass spectra of their cyclic boronic esters⁶⁸⁴ have been examined.

7. General

An article on the mass-spectrometric analysis of g.l.c. effluents has been published,⁹³⁷ together with reviews on g.l.c. in combination with mass spectroscopy⁹³⁸ and with infrared spectroscopy.⁹³⁹ Although combined g.l.c.-m.s. instruments may be purchased, it is often found necessary to adapt existing apparatus. Several papers give details of such modifications; for instance, constructional details for a probe to fit the AEI MS-9 have been published,⁹⁴⁰ a simple heated inlet system has been described,⁹⁴¹ and molecule separators have been discussed.⁹⁴²⁻⁹⁴⁵ Methods for interfacing g.l.c. and m.s. instruments have been reviewed by D. I.

(936) T. Ito, *Agr. Biol. Chem.* (Tokyo), 33, 1217 (1969).

(937) W. H. McFadden, *Advan. Chromatogr.*, 4, 265 (1968).

(938) A. B. Littlewood, *Chromatographia*, 1, 37 (1968).

(939) A. B. Littlewood, *Chromatographia*, 1, 223 (1968).

(940) K. van Cauwenberghe, M. Vandewalle, and M. Verzele, *J. Gas Chromatogr.*, 6, 72 (1968).

(941) C. B. Thomas and B. Davis, *Chem. Ind.* (London), 413 (1969).

(942) R. Ryhage, *Ark. Kemi*, 26, 305 (1967).

(943) M. A. Grayson and C. J. Wolf, *Anal. Chem.*, 39, 1438 (1967).

(944) C. Merritt, Jr., M. L. Bzinet, and W. G. Yeomans, *J. Chromatogr. Sci.*, 7, 122 (1969).

(945) D. R. Black, R. A. Flath, and R. Teranishi, *J. Chromatogr. Sci.*, 7, 284 (1969).

Rees, who gave 35 references.⁹⁴⁶ An improved technique for transferring fractions from a gas chromatograph to a mass spectrometer by use of a heated line and a refrigerated trap has been described by Woolley.⁹⁴⁷ Many other methods of collecting g.l.c. fractions have been described, and representative systems include stainless-steel traps cooled in nitrogen,^{948,949} a U-tube fitted with a wire to act as an electrostatic precipitator,⁹⁵⁰ cigarette filter-tips,⁹⁵¹ and tubes filled with 5 percent of Carbowax on Chromosorb.⁹⁵² The collection of samples specifically for mass spectrometry has been discussed,⁹⁵³ and the use of molecular sieve 5A^{953a} or of activated charcoal^{953b} has been recommended. It has been shown that a lower detector temperature, where practical, may aid in the collection of fractions.⁹⁵⁴ A review by S. G. Perry⁹⁵⁵ on peak identification gives a table of techniques for the collection of fractions, and a paper on the application of g.l.c. to sterols gives 32 references to methods of sample collection.^{955a}

Carbohydrates are relatively nonvolatile and, thus, very simple methods suffice, except for such compounds of low molecular weight as may be encountered after periodate oxidation. We ourselves have obtained very good results with ordinary, melting-point, capillary tubes.⁹⁵⁶ On using an instrument having thermal conductivity detectors and 1/4- or 3/16-inch columns, two or three injections suffice to give weighable quantities of individual components that may be recrystallized, or examined by mass spectrometry and other techniques. A related article⁹⁵³ illustrates the combination of t.l.c.-g.l.c.-m.s., a topic discussed by Kaiser.^{956a}

The problem of bleeding from g.l.c. columns, and its effect on mass

(946) D. I. Rees, *Talanta*, **16**, 903 (1969).

(947) W. D. Woolley, *Analyst*, **94**, 121 (1969).

(948) R. K. Odland, E. Glock, and N. L. Bodenhamer, *J. Chromatogr. Sci.*, **7**, 187 (1969).

(949) K. R. Burson and C. T. Kenner, *J. Chromatogr. Sci.*, **7**, 63 (1969).

(950) J. L. Bloomer and W. R. Eder, *J. Gas Chromatogr.*, **6**, 448 (1968).

(951) K. Hammarstrand, J. M. Juntunen, and A. R. Hennes, *Anal. Biochem.*, **27**, 172 (1969).

(952) A. E. Lipska and F. A. Wodley, *J. Appl. Polym. Sci.*, **13**, 851 (1969).

(953) R. A. Scanlan, R. G. Arnold, and R. C. Lindsay, *J. Gas Chromatogr.*, **6**, 372 (1968).

(953a) M. Cartwright and A. Heywood, *Analyst*, **91**, 337 (1966).

(953b) J. N. Damico, M. P. Wong, and J. A. Sphon, *Anal. Chem.*, **39**, 1045 (1967).

(954) F. Armitage, *J. Chromatogr. Sci.*, **7**, 190 (1969).

(955) S. G. Perry, *Chromatogr. Rev.*, **9**, 1 (1967).

(955a) J. E. Van Lier and L. L. Smith, *J. Chromatogr.*, **36**, 7 (1968).

(956) G. G. S. Dutton and K. B. Gibney, *J. Chromatogr.*, **72**, 179 (1972).

(956a) R. Kaiser, *Chem. Brit.*, **5**, 54 (1969).

spectrometry, has been noted, and it has been pointed out⁹⁵⁷ that "often the column best suited for separation of a given mixture has too high bleeding for use in a combined instrument." In this instance,⁹⁵⁷ separation was best achieved on QF-1, the fractions being collected in moist glass wool, and re-injected in a combined g.l.c.-m.s. instrument equipped with a column of SE-30. Where a column of ECNSS-M is used for separations by g.l.c., this liquid phase is often better replaced by OV-225 for combined g.l.c.-m.s.⁷¹²

On-column silylation of glycols has been discussed,¹³⁶ and it should be noted that such silylation may occur adventitiously. VandenHeuvel and Kuron⁹⁵⁸ found an m/e value of 366 for a phenolic substance when this was separated by g.l.c., although a value of 294 had been expected. However, when this phenolic compound was injected directly into the mass spectrometer (without prior g.l.c.), the anticipated value of 294 was obtained; the discrepancy of 72 mass units was ascribed to on-column silylation.

A mass spectrum recorded while a component is emerging from a gas chromatograph will be distorted if a change in concentration occurs during the scanning. This problem has been discussed,⁹⁵⁹ and a method of ratio recording was proposed that permits scanning to extend over most of the period when a compound is emerging.

XXVI. COLUMN PACKING AND QUANTITATION

Ottenstein⁹⁶⁰ reviewed the various support materials available up to 1963 for use in gas chromatography, and in 1968, he⁹⁶¹ discussed different methods for support deactivation. From the results obtained by using methanol-toluene and hexane-1-pentanol test systems, he concluded that acid washing (AW) or base washing (BW) are ineffective in lessening "tailing," but that treatment with a substituted silane is efficacious, the order being chlorodimethylsilane (DMCS) > hexamethyldisilazane > chlorotrimethylsilane. He found the best deactivation, and thus the best separation, to be achieved by a combination of acid washing and silanization (AW-DMCS). Methods for the deactivation of Chromosorb G have also been reported.⁹⁶² It must, however, be noted that Shaw and Moss⁴¹⁷ found that untreated Chromosorb gives the best separation of

(957) R. Blomstrand and J. Gürtler, *Ark. Kemi*, **30**, 213 (1969).

(958) W. J. A. VandenHeuvel and G. W. Kuron, *J. Chromatogr.*, **38**, 532 (1968).

(959) B. H. Kennett, *Anal. Chem.*, **39**, 1506 (1967).

(960) D. M. Ottenstein, *J. Gas Chromatogr.*, **1**, 11 (1963).

(961) D. M. Ottenstein, *J. Gas Chromatogr.*, **6**, 129 (1968).

(962) J. Martin and L. Gasco, *An. Quím.*, **64**, 775 (1968).

alditol acetates, and this has also been found true with certain methylated alditol acetates.⁷⁹³ Often, silanizing increases the polarity of the liquid phase, and thus increases the retention time.⁹⁶³ It has been calculated that, because of the relatively large size of the trimethylsilyl group, full coverage is obtained when about 60% of the free silanol groups in the silica have reacted.⁹⁶⁴

In 1960, the adsorptive capacity of firebrick was shown⁹⁶⁵ to be considerably lessened by treatment with hexamethyldisilazane, and any adsorption remaining could virtually be completely suppressed by adding 0.1% of poly(ethylene glycol) 400. When a column of SE-30 had been used repeatedly, the peaks became broad,⁹⁶⁶ but the column could be reconditioned by injecting a silicone fluid (Dow-Corning 200).

The efficiency of a column may be improved by subjecting the support to a fluidization process, as, for example, in a stream of nitrogen for 3 hours. This treatment eliminates the "fines," and removes rough edges, giving more nearly spherical particles.⁹⁶⁷ Porous silica beads (Spherosil or Porasil) have been evaluated as nonreactive materials for use in gas-solid chromatography.⁹⁶⁸ Glass beads have been used to support the liquid phase, and, as soda-lime glass is unsuitable (because of surface silanol groups), fused silica or sodium silicate has been recommended.⁹⁶⁹ In addition, ordinary glass beads can adsorb very little liquid phase, and "puddling" may occur with loadings as low as 0.06%; this figure can be increased if surface-etched beads are used.⁹⁷⁰

Porapak and Chromosorb have been compared⁹⁷¹ for the separation of a variety of compounds of low molecular weight without the addition of a liquid phase. Porapak S has been used⁹⁷² for the separation of glycols, and a ceramic material of low density has been used similarly.⁹⁷² The effect of tubing materials on the adsorption of glycols has already been noted;⁶²⁵ anomalous results may also be caused by the borax used as the lubricant in drawing stainless-steel tubes.⁹⁷³ Trifluoroacetates are

(963) W. J. A. VandenHeuvel, W. L. Gardiner, and E. C. Horning, *J. Chromatogr.*, **25**, 242 (1966).

(964) W. J. Eakins, *Ind. Eng. Chem., Prod. Res. Develop.*, **7**, 39 (1968).

(965) J. Bohemen, S. H. Langer, R. H. Perrett, and J. H. Purnell, *J. Chem. Soc.*, 2444 (1960).

(966) L. I. Braddock and N. Marec, *J. Gas Chromatogr.*, **5**, 588 (1967).

(967) C. L. Guillemin, *J. Chromatogr.*, **30**, 222 (1967).

(968) C. L. Guillemin, M. LePage, R. Beau, and A. J. de Vries, *Anal. Chem.*, **39**, 940 (1967).

(969) A. M. Filbert and M. L. Hair, *J. Gas Chromatogr.*, **6**, 150 (1968).

(970) H. L. MacDonell, *Anal. Chem.*, **40**, 221 (1968).

(971) W. R. Supina and L. P. Rose, *J. Chromatogr. Sci.*, **7**, 192 (1969).

(972) N. T. Castellucci and P. R. Eisaman, *J. Gas Chromatogr.*, **6**, 599 (1968).

(973) F. Woutman and F. M. De Ruyter, *J. Gas Chromatogr.*, **4**, 394 (1966).

particularly prone to decomposition on catalytic surfaces, and glass columns have been recommended for them.²¹³ Poor glazing of any ceramic insulation in the gas-flow path may also cause compounds to be adsorbed.⁹⁷⁴

Teflon powders may be used as chromatographic supports, and pre-heating at 300° improves their characteristics.⁹⁷⁵ A simple apparatus for reproducibly packing columns has been described;⁹⁷⁶ a cold-packing method for Teflon is recommended.⁹⁷⁷

The classification and mode of action of various polar phases have been discussed by Chovin,⁹⁷⁸ and Luft and Pin⁹⁷⁹ classified various phases, based on the influence of their polarity on the retention times of hydrocarbons. The theory and principles for choosing and designing selective, stationary phases have been reviewed.⁹⁸⁰ The rate of evaporation of various esters of phthalic acid used as liquid phases has been studied,⁹⁸¹ and the changes in retention time caused by oxidation of nonpolar phases have been examined.⁹⁸² For squalene on Celite, these changes are large, and the retention times may be increased or decreased, depending on the chemical nature of the solute.⁹⁸² Where the stationary phase is sensitive to oxidation, and nitrogen is used as the carrier gas, the life of the column may be prolonged by removing the traces of oxygen found in commercial nitrogen.⁹⁸³ A nonpolar phase that has, apparently, not yet been used in the carbohydrate field is graphitized carbon black coated with 1% of a high-boiling, aromatic compound. Such a liquid phase has a vapor pressure of less than 10^{-4} torr at 300°; thus, the column does not bleed, has a long life, and gives a stable base-line. Excellent separations of hydrocarbons and fatty acid esters have been obtained therewith.⁹⁸³

Verzele and Verstappe⁹⁸⁴ pointed out that care must be exercised in comparing percentages of liquid phases for various packings, because of their different densities. Thus, for columns of equal length, glass beads with 1% of liquid phase are equivalent to Chromosorb with 10%, because

(974) D. R. A. Wharton and M. L. Wharton, *J. Gas Chromatogr.*, **3**, 74 (1965).

(975) A. Saint-Yrieix and C. Lesimple, *Bull. Soc. Chim. Fr.*, 4365 (1967).

(976) R. Villalobos, *J. Gas Chromatogr.*, **6**, 367 (1968).

(977) H. Jowitt, *Chem. Ind. (London)*, 683 (1968).

(978) P. Chovin, *Bull. Soc. Chim. Fr.*, 1800 (1964).

(979) R. Luft and C. Pin, *Compt. Rend. (C)*, **266**, 537 (1968).

(980) S. H. Langer and R. J. Sheehan, *Advan. Anal. Chem. Instrumen.*, **6**, 289 (1968).

(981) N. Petsev and C. Dimitrov, *J. Chromatogr.*, **34**, 310 (1968).

(982) M. B. Evans and J. F. Smith, *J. Chromatogr.*, **28**, 277 (1967).

(983) F. A. Holdinghausen, D. Freitag, W. Ried, and I. Halasz, *Angew. Chem. Int. Ed. Engl.*, **7**, 724 (1968).

(984) M. Verzele and M. Verstappe, *J. Chromatogr.*, **26**, 485 (1967).

the relative densities of the supports are 2.0 and 0.22. They also pointed out that glass beads bearing 1% of a liquid phase are difficult to handle unless the liquid phase is solid at room temperature (for example, Carbowax 20 M). The performance of any column is determined by the efficiency with which it is packed, and one guide to this efficiency is the weight of packing per unit of column length. Table XXXII (see p. 105) shows, for the more common packings, the recommended weights for different column sizes.⁹⁸⁵

Good results have been obtained in preparative g.l.c. with gradient-loaded columns containing 40% of liquid phase at the entry, decreasing to 10% at the exit.⁹⁸⁶ Better resolution was obtained than with a linearly packed column. Similar results were obtained in the separation of glycol ethers, but the column loading was increased from 2% at the entry to 10% at the exit.⁹⁸⁷ When large samples are injected onto a column, the efficiency (in terms of theoretical plates) falls sharply, and the stationary phase is physically washed off the beginning of the column and may be deposited farther along.⁹⁸⁸ For this reason, it is wise to avoid injecting samples in such solvents as pyridine, which may readily dissolve the liquid phase.

The most detailed study thus far made of the influence of column packings and liquid phases on the separation of trimethylsilyl derivatives of sugars is that by Ellis,²⁸⁹ although Bauer and coworkers had earlier reported similar studies.³⁷⁹ A comparison of polar and nonpolar columns has been made by Farshtchi and Moss.²⁹⁰

The choice of carrier gas in preparative g.l.c. has been discussed by Verzele,⁹⁸⁹ and, in a study of quantitative g.l.c. using thermal conductivity detectors, Kebbekus and coworkers⁹⁹⁰ concluded that the carrier gas should be chosen to suit the sample. Negative peaks recorded by thermal conductivity detectors have been attributed⁹⁹¹ to impurities in the carrier gas.

The selection of a liquid phase for a particular separation has deliberately not been discussed in this Section, because the entries in the Tables provide guidance on this point. It suffices to note that liquid phases having improved thermal stability are now available, and in the OV

(985) "Gas Chrom. Newsletter," Applied Science Laboratories, State College, Pennsylvania, 1970, Vol. 11, No. 1.

(986) R. C. Duty, *J. Gas Chromatogr.*, **9**, 193 (1968).

(987) M. Singlair and J. Dykyj, *Collect. Czech. Chem. Commun.*, **34**, 767 (1969).

(988) L. Mikkelsen, F. J. Debbrecht, and A. J. Martin, *J. Gas Chromatogr.*, **4**, 263 (1966).

(989) M. Verzele, *J. Chromatogr.*, **15**, 482 (1964).

(990) B. B. Kebbekus, M. H. Barsky, R. T. Rossi, and J. Jordan, *J. Amer. Chem. Soc.*, **88**, 2398 (1966).

(991) G. Castello and G. D'Amato, *J. Chromatogr.*, **32**, 625 (1968).

series, for example, the polarity of the phase increases from OV-1 to OV-225.

Review articles have discussed the relative efficiencies of g.l.c. and gel filtration,⁹⁹² the optimal conditions in g.l.c. analysis,⁹⁹³ preparative g.l.c.,⁹⁹⁴ flow programming,⁹⁹⁵ and the quantitative interpretation of data.⁹⁹⁶ Recommended terms and relationships for use in gas chromatography have been published,⁹⁹⁷ the use of pressure programming has been discussed,⁹⁹⁸ and a statistical analysis has been made of the techniques of quantitative g.l.c.⁹⁹⁹ It was concluded that the main errors arise in measuring the amount of sample, evaluating the peak size, and determining molar response-factors. Apparatus errors were considered to be relatively insignificant. The same article⁹⁹⁹ is particularly useful for the references given to earlier work on quantitative problems. A symposium on quantitative, gas-liquid chromatography dealt in particular with sources of error, and errors in manual-integration techniques.¹⁰⁰⁰

The types of thermal conductivity detectors and their mode of operation have been reviewed by Lawson and Miller.¹⁰⁰¹ Fowlis and co-workers¹⁰⁰² examined the argon and flame-ionization detectors commercially available, and Cremer¹⁰⁰³ discussed thermionic and electrochemical detectors for halogen and sulfur. Goedert and Guiochon¹⁰⁰⁴ examined the reproducibility of the response of a catharometer, and showed that this depends on the mass flow-rate of the carrier gas, the pressure of the carrier gas in the measuring cell, the bridge current, the detector temperature, and the mass of the sample. In certain cases, the detector temperature may be critical. For example, in the separation of cycloamyloses as their dimethylsilyl ethers, Beadle¹³³ noted that a small decrease in the detector temperature gave excessively broad peaks. Devaux and Guiochon studied the variation of the response factor of electron-capture detectors.¹⁰⁰⁵ Sherman and Goodwin⁵⁷⁹ found that this

(992) J. C. Giddings, *Anal. Chem.*, **39**, 1027 (1967).

(993) I. Halasz and E. Heine, *Advan. Anal. Chem. Instrumen.*, **6**, 153 (1968).

(994) D. T. Sawyer and G. L. Hargrove, *Advan. Anal. Chem. Instrumen.*, **6**, 325 (1968).

(995) R. P. W. Scott, *Advan. Anal. Chem. Instrumen.*, **6**, 271 (1968).

(996) H. W. Johnson, Jr., *Advan. Chromatogr.*, **5**, 175 (1968).

(997) American Society for Testing and Materials, E 355 (1968).

(998) J. D. Kelley and J. Q. Walker, *J. Chromatogr. Sci.*, **7**, 117 (1969).

(999) P. Boček, J. Novák, and J. Janák, *J. Chromatogr.*, **42**, 1 (1969).

(1000) J. M. Gill and H. W. Habgood, *J. Gas Chromatogr.*, **5**, 595 (1968).

(1001) A. E. Lawson and J. M. Miller, *J. Gas Chromatogr.*, **4**, 273 (1966).

(1002) I. A. Fowlis, R. J. Maggs, and R. P. W. Scott, *J. Chromatogr.*, **15**, 471 (1964).

(1003) E. Cremer, *J. Gas Chromatogr.*, **5**, 329 (1967).

(1004) M. Goedert and G. Guiochon, *J. Chromatogr. Sci.*, **7**, 323 (1969).

(1005) P. Devaux and G. Guiochon, *J. Gas Chromatogr.*, **5**, 341 (1967).

type of detector is particularly suitable for keto compounds; *myo*-inosose-2 may be detected with high sensitivity, whereas sugars have only about 0.002 of this sensitivity.

The use of molar response-factors (m.r.f.) for individual components of a mixture has long been an area of controversy. Early workers tended to assume that the response of isomeric, or similar, compounds was identical, especially for flame-ionization detectors. The results of later investigations^{1008,1007} using improved instrumentation discredited these ideas, and made plain that, for accurate quantitation, m.r.f. values should *always* be obtained.^{36,114,116,171,208,218a,412,608,1008,1009} Lewicki and Edwards^{819a} showed that the presence or absence of a methyl group on O-6 of glucose has a profound effect on the m.r.f., and, thus, the methyl glycosides of 2,3,4- and 2,3,6-tri-*O*-methyl-*D*-glucose give different responses. It should be noted that these values may vary with the sample treatment and operating parameters, and should, therefore, be determined by each individual, using the exact experimental conditions to be employed for subsequent analyses. This conclusion can best be emphasized by quoting Herb and colleagues:¹⁰¹⁰ "Precise analysis requires application of empirical correction factors. These factors should be determined under the same operating conditions used in the analysis and preferably with a known mixture of compounds which approximate the percentage to be found in the sample to be analyzed." Gee and Walker⁶⁸⁸ pointed out that the m.r.f. values for tri- and tetra-*O*-methylhexosides are similar when columns bearing a low percentage of liquid phase are used, but that, at higher concentrations, the values vary greatly, depending on the column conditions. Verhaar and de Witt¹⁶⁶ showed that, for the silyl derivatives of hydroxy acids, the m.r.f. values were reproducible with a column of PO-17, but fluctuated widely with a column of PPE-5. This behavior was attributed to interaction between the silylated compound and an incompletely deactivated support. In connection with the estimation of alditol acetates, Bowker and Turvey⁴¹² commented on the molar responses.

B. D. Smith and Bowden¹⁰¹¹ presented a method for the prediction of response factors, and a computer program for their calculation from ex-

(1006) W. A. Dietz, *J. Gas Chromatogr.*, **5**, 68 (1967).

(1007) R. Gelin and F. Godot, *Bull. Soc. Chim. Fr.*, 3096 (1966).

(1008) A. J. Sheppard, S. A. Meeks, and L. W. Elliott, *J. Gas Chromatogr.*, **6**, 28 (1968).

(1009) A. J. Sheppard, A. R. Prosser, and L. W. Elliott, *J. Gas Chromatogr.*, **6**, 34 (1968).

(1010) S. F. Herb, P. Magidman, and R. W. Riemenschneider, *J. Amer. Oil Chem. Soc.*, **44**, 32 (1967).

(1011) B. D. Smith and W. W. Bowden, *Anal. Chem.*, **36**, 82 (1964).

perimental data has been published.⁸⁸⁰ Stephen and coworkers⁷⁷⁶ showed that, for a series of methylated sugars, there is a correlation between m.r.f. values and the logarithm of the retention time. It has been stated¹⁰⁰⁶ that m.r.f. values are independent of the temperature, the carrier gas, or the flow rate, although it has been shown¹⁰¹² that the sensitivity of the detector and the area of the peak are inversely proportional to the rate of gas flow.

In certain instances, mixtures of silylated or methylated sugars have been injected in carbon disulfide, a compound generally considered to give a low, or zero, response by flame ionization. The response has been shown¹⁰¹³ to be dependent on the flow rate and the geometry of the detector. In a study¹⁰¹⁴ on the effect of hydrogen and carrier-gas flow-rates on m.r.f. values determined by flame ionization, it was found that the values for all oxygen-containing compounds studied were strongly dependent on the flow rates of both the nitrogen and the hydrogen. Marmion¹⁰¹⁵ warned that, although the sensitivity of flame-ionization detectors is dependent on the gas flow-rate, the change in sensitivity may consist in an increase for some compounds but a decrease for others.

During precise determinations of m.r.f. values, Girling and co-workers¹⁰¹⁶ observed that the repeatability of the results was governed by the peak widths in successive chromatograms. These variations were attributed to irregularities in the chart movement, and, of seven recorders tested, the worst showed a variation of $\pm 3.65\%$, and the best, $\pm 0.033\%$.

Seven methods involving measurement of peak height for determining areas under chromatographic curves have been compared and shown to be of equal precision.¹⁰¹⁷ A commercial publication¹⁰¹⁸ concluded that digital integration is 3 to 10 times as precise as cutting and weighing, triangulation, or planimetry, and is also significantly faster. A method for the elimination of errors caused by baseline drift has been published,¹⁰¹⁹ and it has been pointed out¹⁰²⁰ that, when a component is present in low concentration, its retention time may be lengthened. It has

(1012) F. Damm, A. Pichler, and S. Libs, *Bull. Soc. Chim. Fr.*, 3020 (1967).

(1013) M. Dressler, *J. Chromatogr.*, **42**, 408 (1969).

(1014) O. Hainová, P. Boček, J. Novák, and J. Janák, *J. Gas Chromatogr.*, **5**, 401 (1967).

(1015) D. H. Marmion, in "Biomedical Applications of Gas Chromatography," H. A. Szymanski, ed., Plenum Press, New York, N. Y., 1968, Vol. 2, p. 179.

(1016) G. W. Girling, A. R. Gigg, and M. R. Heley, *J. Chromatogr.*, **31**, 525 (1967).

(1017) R. B. Mefferd, Jr., R. M. Summers, and J. D. Clayton, *J. Chromatogr.*, **35**, 469 (1968).

(1018) "Previews and Reviews," Varian Aerograph, Walnut Creek, California, 1967, No. 7.

(1019) J. D. Wilson and C. A. J. McInnes, *J. Chromatogr.*, **19**, 486 (1965).

(1020) R. G. Ackman, *J. Gas Chromatogr.*, **3**, 15 (1965).

also been shown that, even when two components differ in retention time by 30%, a trace constituent may appear only as a shoulder.¹⁰²¹ Analysis of such poorly resolved components may be facilitated by the use of a derivative detector.¹⁰²²

The factors impairing reproducibility of sample injection and of g.l.c. temperature cycles have been discussed by Pitt¹⁰²³ and by Ebing.¹⁰²⁴ Hautala and Weaver¹⁰²⁵ showed that changes in column temperature or in the rate of heating change the peak shapes, and thus lower the precision. Hancock and Cataldi¹⁰²⁶ recommended raising the temperature in steps, instead of linearly, in order to minimize long baselines. "Anomalous" adsorption of sample in the injection port has been noticed,¹⁰²⁷ and an improved technique of injection for repetitive sampling, where accuracy depends on the ability to inject exact sample-volumes repeatedly, has been described.¹⁰²⁸

Brandt and Lands¹⁰²⁹ pointed out that an injected sample diffuses, and, consequently, separation does not begin from a point source but from a band. Correct calculation of peak areas by retention time \times peak height therefore requires introduction of a correction factor, use of which gives results comparable with the (more time-consuming) triangulation method. They further showed that a plot of peak width at half height against retention time gave a straight line that did not pass through the origin, indicating an imaginary injection-point occurring several seconds earlier than the real injection-point.

XXVII. TABLES

The general format of the following Tables parallels that used in Part I (see Section XV in Vol. 28, p. 101).

Sugars are referred to by standard abbreviations (for example, Glc, glucose); some less-common abbreviations are Abe, abequose; Tyv, tyvelose; Par, paratose. The same abbreviation is used for a free sugar and a derivative thereof. Thus, Glc in Table XVI (see p. 56) refers to methyl glucoside, in Tables XXII and XXIII (see pp. 83 and 86) to glucose, and in Table XXVI (see p. 89) to glucitol.

- (1021) R. L. Levy, *J. Chromatogr.*, **34**, 249 (1968).
- (1022) T. Kambara and K. Saitoh, *J. Chromatogr.*, **35**, 318 (1968).
- (1023) P. Pitt, *Chromatographia*, **1**, 252 (1968).
- (1024) W. Ebing, *Chromatographia*, **2**, 74 (1969).
- (1025) E. Hautala and M. L. Weaver, *Anal. Biochem.*, **30**, 32 (1969).
- (1026) H. A. Hancock and A. Cataldi, *J. Gas Chromatogr.*, **5**, 406 (1967).
- (1027) M. Krejci, K. Hana, and M. Roudna, *J. Chromatogr.*, **41**, 145 (1969).
- (1028) H. E. Sparks, *J. Gas Chromatogr.*, **6**, 410 (1968).
- (1029) A. E. Brandt and W. E. M. Lands, *Lipids*, **3**, 178 (1968).

Certain of the liquid phases are designated in the Tables by contractions, as in Part I: BDS, butanediol succinate; EGA, ethylene glycol adipate; EGS, ethylene glycol succinate; NPGA, neopentyl glycol adipate; NPGS, neopentyl glycol succinate; and NPGSE, neopentyl glycol sebacate. When a component of a mixture is shown in parentheses, for example: Ara 2,3,5; 2,3; (2), this indicates that the component was not detected by g.l.c. but by an alternative method such as paper chromatography.

TABLE XIV
Representative Methylations Using Sodium Hydride

<i>Compound</i>	<i>References</i>
Arabinan	1030,1030a,1031,1031a
Xylan	685,701,753,762,1032-1035
Galactans	
Arabinogalactan	1031,1036
Mannofucogalactan	462
Fucogalactan	464,678
Glucans	718,757,763,764,1037,1038
Dextran	749
Glycogen	758
Starch	775
Fucoglucuronoglucan	760
Fucoxyloglucan	1036
Glucofructan	363,1039
Glucuronoxylifucan	770
Gum	769,775,1040
Mannans	678,838
Glucomannan	793,1033
Fucoxylomannan	463
Galactoglucomannan	1041
Pectin	104,786,1036
Lipopolysaccharide	16,432,435,437-440a,443, 484a,712,720,727-730, 781b,832,883,922,1042- 1046
with methyl iodide- <i>d</i> ₃	445,713,787
with ethyl iodide	765
micro	444
Ovomucoid	758
Glycolipid	758
Capsular	720,760,861,862,1047-1055
Ceramide (micro)	1056
Monosaccharides	231,533,771,782
Aldobiouronic acids and disaccharides	767,769,773,781a,845,884, 1057

TABLE XV
Model Studies on Methyl Ethers

<i>Sugar</i>	<i>Derivative</i>	<i>References</i>
Arabinose	free reducing sugars	798,803
	methyl glycosides	739,781c,803
	trimethylsilyl ethers of methyl glycosides	379
	acetylated methyl glycosides	798
	alditol acetates	798,831
	lactones	803
Xylose	trimethylsilyl derivatives of free sugars	601,852
	trimethylsilyl ethers of methyl glycosides	601
	methyl furanosides	800,801
	alditol acetates	799,831
	acetylated diethyl dithioacetals	799
	acetylated nitriles	799
Glucose	trimethylsilyl derivatives of free sugars	402,654,819
	acetates of free sugars	817,826a
	methyl glycosides	688,818,819a
	acetylated methyl glycosides	797,816,847
	alditol acetates	816,820,831,832
	alditol trifluoroacetates	820
	alditol trimethylsilyl ethers	820
	septanosides and unsaturated derivatives	811-815
Galactose	acetates of free sugars	826a
	methyl glycosides	379,804
	methyl glycosides of Gal and GalA	806
	trimethylsilyl ethers of methyl glycosides	379
	trimethyl ethers	717,805
	alditol acetates	831,834-837
	septanosides	807
Mannose	trimethylsilyl derivatives of free sugars	824,1058
	acetates of free sugars	826a
	methyl glycosides	782,824,824a
	trimethylsilyl ethers of methyl glycosides	782,790,794a, 824,824a,838, 1058
	lactones	640,1058
	alditol acetates	831
	di- and mono-methyl ethers	641
Rhamnose	lactones	641
Fructose	methyl glycosides	479,688
Sucrose and other di- and tri-saccharides	methyl glycosides	688

TABLE XV (Continued)

<i>Sugar</i>	<i>Derivative</i>	<i>References</i>
Ara, Xyl, Rha, Gal, Glc, Man	fully methylated glycosides	231
Ery, Ara, Xyl, Rha, Gal, Glc, Man	fully methylated alditols	231
Ara, Fru, Glc, Gal	fully methylated glycosides and aldehydo forms	808-810
2-Amino-2-deoxyglucose	methyl glycosides	716,717
	alditol acetate	830a
2-Amino-2-deoxygalactose	methyl glycosides	716,717
	alditol acetates	716,717
2-Deoxy-2-(methylamino)- glucose and 2-deoxy-2- (methylamino)galactose	trimethylsilyl ethers and acetates of alditols	827
Cyclitols	trimethylsilyl ethers	1059
	methyl ethers	254,739

TABLE XVI
Methyl O-Methylglycosides

Compounds separated			Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups	Column of				
A. Arabinans and arabinogalactans						
Ara	2,3,5; 2,3; 2	5% SE-30			beet arabinan	1060
Ara	2,3,5; 2,3; (2)	5% NPGA	165		lemon-peel pectin	1031
		10% Carbowax 20 M	190		soybean-cotyledon meal	1031a
Ara	2,3,5; 2,3; 3,5; (2,5; 2)	10% NPSG	168		synthetic arabinan	875,1030
Ara	2,3,4; 2,3,5; 2,3; 2 (MeAra + Ara)	15% BDS	175		<i>Brassica sinapis alba</i> (mustard seed)	1061
		10% poly(phenyl ether)	200			
Ara	2,3,5; 2,3; 2; (2,5; 3,5; 2; Gal 2,3,6; 2,3)	15% BDS			<i>Mycobacterium bovis</i>	870
Ara	2,3,5 lactone	7% Versamid	155		<i>Centrosema plumari</i>	869
	Gal 2,3,4,6 lactone (2,3,4; 2,3,6; 2,4,6; 2,3; 2,4)					
Ara	2,3,5; 2,5; 3,5	5% NPGA	150		<i>Araucaria bidwilli</i>	1062
Gal	2,3,4,6 (Ara 2)	10% poly(phenyl ether)	200			
Ara	2,3,5; 2,5; 3,5 lactones	5% NPGA	150			
Ara	2,3,5; 2,3; 2,5; 2	15% BDS	150-240		<i>Mycobacterium tuberculosis</i>	1063
Gal	2,3,6					
Ara	2,3,5; 2,5; 3,5	14% EGS	155		<i>Watsonia pyramidata</i>	105
Gal	2,3,4,6; 2,3,6 (Ara 3; Xyl 3; and Xyl)					
Ara	2,3,5; 2,3; (2)	5% NPGA	165		lemon-peel pectin	1031
Gal	2,3,4,6; 2,3,6; 2,6	5% XE-60	125,150			
Ara	2,3,4; 2,3,5				mountsin and european larch	1063a
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4 +4 unknowns (GlcA 2,3,4; Gal 2)					
Ara	2,3,5; 2,3	5% NPGA	165		<i>Acer pseudoplatanus</i> (sycamore)	1036
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4	15% EGA	175			
		10% Carbowax 20 M	185			

Ara	2,3,4; 2,3,5; 2,5	10% EGS			<i>Larix laricina</i> (tamarack, compression wood)	850
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4					
Ara	2,3,5; 2,3; 2,5; (2)	5% NPGA	165		lemon-peel pectin	1031
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4	5% XE-60	125,150			
Ara	2,3,4; 2,3,5; 2,3; 2,4; 2,5; 3,4; 3,5	3% NPGA	150		mesquite gum, arabinan chains	803
Gal	2,3,4,6; 2,3,4	5% EGA	175			
Ara	2,3,4; 2,3,5; 2,3; 2,5; 2	10% BDS	175		<i>Larix leptolepis</i> (Japanese larch)	751
		10% poly(phenyl ether)	200		tritylated arabinogalactan	
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4; 2,6; (2)	5% NPGA	150			
		5% XE-60	125			
<i>B. Xylans</i>						
Xyl	2,3,4; 2,3 (Glc 2,3,4,6; 2,3,6 from contaminating starch)	14% EGS	155		<i>Watsonia pyramidata</i>	105
Xyl	2,3,4; 2,3 (2; 3)	10% EGS	110-150	2	<i>Helianthus annuus</i> (sunflower)	1064
Xyl	2,3,4; 2,3; 2,4	poly(phenyl ether)	140		<i>Rhodymenia palmata</i> (red seaweed)	748
Xyl	2,3,4; 2,3; 2	5% BDS	150		<i>Populus monilifera</i>	605
		Carbowax 6000	165			
Xyl	2,3,4; 2,3; 2; 3	20% Apiezon M	150		<i>Populus tremuloides</i> (aspen)	599
		20% BDS	150			
Ara	2,3,5	10% EGS			<i>Larix laricina</i> (tamarack, compression wood)	848
Ara	2,3,5	10% EGS	110-150	2	<i>Picea abies</i> (Norway spruce)	601
Xyl	2,3,4; 2,3					
Xyl	2,3,4; 2,3	20% Apiezon M			<i>Pteridium aquilinum</i> (bracken)	1064a
Glc	2,3,4	20% Carbowax 20 M				
(Xyl)	2; 3)					
Xyl	2,3,4; 2,3; 3	10% poly(phenyl ether)			<i>Eucalyptus globulus</i>	1032
Glc	2,3,4				<i>Betula verrucosa</i> (reduced xylans)	
Ara	2,3,5	10% poly(phenyl ether)	200		<i>Avena sativa</i> (oat-leaf hemi-cellulose)	761
Xyl	2,3,4; 2,3					
Gal	2,3,4,6					
(Xyl)	2; 3)					

(Continued)

TABLE XVI (Continued)

Compounds separated			Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups	Column of				
Ara Xyl Glc	2,3,5 2,3,4; 3 2,3,4	5% BDS	120		<i>Zea mays</i> (corn stalk and leaves)	701
Xyl	2,3,4; 2,3; 2; 3	10% EGS	110-150	2	<i>Tilia americana</i> L. (basswood)	846
Xyl Glc	2,3,4; 2,3; 2; 3 2,3,4	10% poly(phenyl ether)			<i>Bombax malabaricum</i> (reduced xylan)	685
Xyl Glc	2,3; 3,4; 2; 3 2,3,4	10% BDS	185		<i>Salix alba</i> (white willow)	752
Ara Xyl	2,3,5; 3,5 2,3,4; 2,3; 2	14% EGS 15% BDS	160 160		<i>Cynodon plectostachyus</i> (giant star grass) <i>Setaria sphacelata</i>	753
Xyl Ara	2,3,4; 2,3; 2; 3 2,3,5	10% EGS 10% NPGSE 10% Apiezon M	150-180		<i>Teuga canadensis</i>	600
Xyl Gal	2,3,4; 2,3; 3,4; 2; 3 2,3,4 (Glc 2,3,4)	10% BDS 10% poly(phenyl ether) 3% NPGA	175 200 150		soybean hulls	1065
Ara Xyl Glc Gal	2,3,5 2,3,4; 2,3; 2 2,3,4,6; 2,3,6; 2,4,6 2,3,4,6	3% ECNSS-M 10% poly(phenyl ether)			<i>Avena sativa</i> (oat delignification)	1034
Ara Xyl Glc Gal	2,3,5; 2,3; 3,5 2,3,4; 2,3; 2; 3 2,3,4 2,3,4,6	3% ECNSS-M 10% poly(phenyl ether) 8% BDS			<i>Avena sativa</i> (galactoarabinoxylan from oat)	1035
Xyl Ara Gal GlcA	2,3,4; 2,3; 2; 3 2,3,5; 2,5; 3,5 2,3,4,6; 2,3,6 2,3,4	14% EGS	155		<i>Walsonia versveldii</i>	1066
C. Galactans						
Gal	2,3,4,6; 2,4,6 (2,3,4; 2,3,6)	20% EGA 10% Carbowax 6000	200 164		ceramide dihexoside of erythrocytes	805

Gal	2,3,4,6; 2,3,5,6; 2,3,6	15% poly(phenyl ether)	190	carboxyl-reduced pectic acid from sunflower	1067
Gal	2,3,4,6; 2,3,6; 2; 3	10% EGS 5% SE-52		<i>Larix laricina</i> (tamarack, acidic galactan)	858
Gal	2,3,6; 2,4,6 3,6-anhydro-2-Me	BDS	175	Porphyran	690
Gal Glc	2,3,4,6; 2,3,6 2,3,6	20% EGA	190	ceramide di- and tri-hexoside of human kidney	689
Gal GalA	2,3,4,6; 2,3,5,6 2,3	5% NPGS	167	<i>Zosteraceae</i> pectin	786
Gal	2,3,4,6; 2,4,6; 2,4; 4,6 3,6-anhydro-2-Me (3)	15% EGA	175	λ and μ carrageenans	1068
Gal	2,3,4,6; 2,3,6; 2,4,6; 2,6; 4,6	10% poly(phenyl ether)	200	λ carrageenan	741
Gal	2,3,4,6; 2,3,5,6; 2,3,4; 2,3,6; 2,4,6; 2,3	5% NPGS	167	<i>Panax ginseng</i> pectin	104
Gal Rha	2,3,4,6; 2,3,6 2,3,4; 3,4	15% BDS 10% poly(phenyl ether)	175 200	carboxyl-reduced (partially) pectic acid from tobacco	1069
Ara Gal Rha	2,3,5; 2,3 2,3,4,6; 2,3,6; 2,6 3,4; 3	10% BDS 10% poly(phenyl ether) 3 or 5% NPGA	175 200 125-150	carboxyl-reduced, lemon-peel pectin	1070
Xyl Gal	2,3 2,3,4,6; 2,3,6	10% Apiezon M 10% EGS		<i>Picea rubens</i> (galactan from red-spruce compression-wood)	571
GleA GalA	1,4-anhydro-2,3,6- 2,3,4 2,3,4				
Xyl Glc	2,3,4; Me ₂ 2,3,4,6; 2,3,4 or 2,3,6; 2,4,6	15% BDS 15% EGA	175 185	<i>Laurencia pinnatifida</i> (red seaweed)	1064
Gal	2,4,6; 2,3; 2,6; 4,6; 2				
<i>D. Glucans</i>					
Glc	2,3,4	15% Carbowax 20 M	175	glycolipids of wheat flour	1071
Glc	2,3,4-Me ₂ -6-palmitoyl	10% SE-30 3% SE-52		6-O-palmitoylglucose	354
Glc	2,3,4,6	10% EGS	210	sophorosides	592
Glc	2,3,4,6; 2,3,4	5% NPGS	180	<i>Gyrophora esculenta</i>	1037,1038
Glc	2,3,4,6; 2,3,6	3% Carbowax 20 M		cellulose	794

(Continued)

TABLE XVI (Continued)

Compounds separated		Column of	Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups					
Glc	2,3,4,6; 2,4,6	15% BDS	180		<i>Alcaligenes faecalis</i>	1072
Glc	2,3,4,6; 2,3,6	10% Carbowax 40 M	165		<i>Bacillus licheniformis</i>	1073
Glc	2,3,4,6; 2,3,4	10% EGS	150-190		<i>Corynebacteria</i> and <i>Mycobacteria</i> acylglucoses	773
Glc	2,3,4,6; 2,3,6; 2,4,6	20% Apiezon M	150		<i>Monodus subterraneus</i>	1074
		15% BDS	155,175			
		10% poly(phenyl ether)	200			
Glc	2,3,4,6; 2,3,4; 2,4	15% BDS	175		dextran from <i>Leuconostoc mesenteroides</i>	749
		15% poly(phenyl ether)	165			
Glc	2,3,4,6; 2,4,6; 2,4	15% BDS			<i>Phaeodactylum tricornutum</i>	1075
		10% poly(phenyl ether)	175			
Glc	2,3,4,6; 2,3,6	2% Carbowax 20 M	155		<i>Mycobacterium phlei</i> and <i>M. tuberculosis</i>	689
	2,6	2% NPGS	155,202			
Glc	2,3,4,6; 2,3,4; 2,4,6; (2,4)	10% EGS	200		<i>Piricularia oryzae</i>	1075a
Glc	2,3,4,6; 2,3,4; 2,4,6 (Me ₂ + Me)				laminaran	1076
Glc	2,3,4,6; 2,4,6; Me ₂	10% EGS			<i>Poria cocos</i> (pachyman)	797
					<i>Larix laricina</i> (tamarack compression-wood)	847,847a
Glc	2,3,4,6; 2,3,6; Me ₂	20% Apiezon M	150		various green seaweeds	1077
		20% BDS	150			
Glc	2,3,4,6; 2,3,6; Me ₂	15% BDS	180		<i>Torulopsis ingeniosa</i>	760
		10% Carbowax 6000	180		(for dimethylglucoside)	
Glc	2,3,4,6; 2,3,4; 2,4,6; 2,4	15% BDS	180		<i>Aureobasidium pullulans</i>	1078
		15% poly(phenyl ether)	180			
Glc	2,3,4,6; 2,3,4; 2,4,6; 3,4	10% poly(phenyl ether)	200		<i>Leuconostoc mesenteroides</i> dextrans	757a
Glc	2,3,4,6; 2,3,4; 2,4,6; 2,4	10% NPGSE			<i>Trichophyton</i> spp.	1079
Glc	2,3,4,6; 2,3,4; 2,4,6; 2,4	10% NPGS	225		<i>Microsporum quinceanum</i>	584
Glc	2,3,4,6; 2,3,4; 2,3,6 Me 3-Me-glycerate	10% Carbowax			<i>Mycobacterium phlei</i>	789

Glc	2,3,4,6; 2,3,4; 2,3,6; 2,3; 2,4	Carbowax 20 M treated with 2-nitroterephthalic acid		model	819a
Glc	2,3,4,6; 2,3,6; 2,4,6; 2,4; 4,6	10% NPGS	175	<i>Phytophthora cinnamomi</i>	590
Glc (Glc	2,3,4,6; 2,3,4; 2,4,6; 2,3; 2,4 2)	10% NPGSE		<i>Candida albicans</i> and <i>C. parapsilosis</i>	1080
Glc	2,3,4,6; 2,3,4; 2,4,6; 2,3; 2,4; 3,4	3% NPGA	160	<i>Leuconostoc mesenteroides</i> dextran	52
		3% NPGA	175		
		(or 10% NPGA)	175		
Glc	2,3,4,6; 2,3,6	10% Carbowax 6000	155	<i>Serratia marcescens</i>	676
Rha	2,4	10% NPGS	175		
		15% poly(phenyl ether)	200		
Glc	2,3,4,6; 2,3,4; 2,3,6 2,3,4,6,7; 3,4,6,7-D-glycero-D-manno-heptose 2,3,4,6,7-L-glycero-D-manno-heptose	as above		<i>Serratia marcescens</i>	676
Glc	2,3,4,6-Pr _a 3-Me-2,4,6-Pr _a (6-Me-2,3,4-Pr _a)	13% EGS	174	<i>Mycobacterium phlei</i>	654
<i>E. Gums</i>					
Ara	2,3,4; 2,3,5; 2,5; 3,4; 3,5	15% BDS	175	<i>Acacia arabica</i> original gum, de-graded and Smith residues	653
		15% EGA	160		
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4				
Rha	2,3,4				
Glc	2,3,4,6				
GlcA	2,3,4				
(Gal	2,6; 2 and Ara 4)				
Ara	2,3,4; 2,3,5	15% BDS	175	<i>Acacia drepanolobium</i> autohydrolysis and Smith residues	1081
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4; 2,6	15% EGA	160		
GlcA	2,3,4				
(Gal	2)				
Ara	2,3,4; 2,3,5; 2,5; 3,5; 3,4	15% BDS	175	<i>Acacia drepanolobium</i>	1082
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4	15% EGA	160		1083
Rha	2,3,4				
GlcA	2,3,4				
(Ara	4 and Gal 2)				

(Continued)

TABLE XVI (Continued)

Compounds separated			Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups	Column of				
Ara	2,3,4; 2,3,5; 2,3; 2,5; 3,4; 3,5	14% EGS	155		<i>Acacia karroo</i>	1084
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4;	6% EGS	155			
	2,6	20% EGS	150,190			
Rha	2,3,4					
GlcA	2,3,4; 2,3				<i>Acacia laeta</i>	1085
(Ara	3; Gal 2)					
Ara	2,3,4; 2,3,5; 2,5	15% BDS	175			
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4	15% EGA	160			
Rha	2,3,4					
GlcA	2,3,4; 2,3					
(Gal	2)				<i>Acacia mearnsii</i> original gum, carboxyl-reduced gum, auto- hydrolysis and Smith residues	96
Ara	2,3,4; 2,3,5; 2,3; 2,5	15% BDS	175			
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4	10% poly (phenyl ether)	200			
Rha	2,3,4	3% NPGA	150			
Glc(A)	2,3,4; 2,3				<i>Acacia nubica</i> Benth original gum, degraded and Smith residues	637
Ara	2,3,4; 2,3,5; 2,5; 3,5	15% BDS	175			
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4;	15% EGA	160			
	2,6					
GlcA	2,3,4				<i>Acacia podalyriaefolia</i> <i>Acacia elata</i>	106 460
(Gal	2)					
Ara	2,3,5; 2,5; 3,5	14% EGS	155			
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,3;					
	2,4; 2,6				<i>Acacia senegal</i> Willd original gum and Smith residues	1086
Rha	2,3,4					
Ara	2,3,4; 2,3,5; 2,5	15% BDS	175			
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4; 2,6	15% EGA	160			
Rha	2,3,4					
GlcA	2,3,4; 2,3					
(Gal	2)				<i>Acacia senegal</i>	1087
Ara	2,3,4; 2,3,5; 2,5	15% BDS	175			
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4; 2,6	15% EGA	160			
Rha	2,3,4					
GlcA	2,3,4; 2,3					
(Gal	2)					

Ara	2,3,4; 2,3,5; 2,5; 3,5; 3,4			<i>Acacia seyal</i>	1087a
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4				
Rha	2,3,4				
GlcA	2,3,4; 2,3				
Glc	2,3,4,6 (after redn. and meth.)				
(Ara	4)				
Ara	2,3,4; 2,3,5; 2,3;	14% EGS	155	Eight species of <i>Acacia</i>	1088
	2,5; 3,4; 3,5			<i>A. mearnsii</i> , <i>A. karroo</i> , <i>A.</i>	
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4;			<i>cyanophylla</i> , <i>A. decurrens</i> , <i>A.</i>	
	2,6			<i>pycnantha</i> , <i>A. elata</i> , <i>A. po-</i>	
Rha	2,3,4			<i>dalyriaefolia</i> , <i>A. giraffae</i>	
GlcA	2,3,4; 2,3				
	(MeGal)				
Ara	2,3,5; 2,5; 3,5	14% EGS	155	<i>Agave americana</i>	776
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4; 2,6				
Rha	2,3,4				
GlcA	2,3,4; 2,3				
Ara	2,3,4; 2,3,5; 2,5	10% BDS	175	<i>Araucaria bidwilli</i> original gum	783
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4; 2,6	10% poly(phenyl ether)	200	and Smith residues	
Rha	2,3,4				
Glc(A)	2,3,4				
(Gal	2)				
Ara	2,3,4; 2,3,5; 2,5	5% NPGA	150	<i>Araucaria bidwilli</i> original gum,	1062
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4	10% poly(phenyl ether)	200	carboxyl-reduced gum	
Rha	2,3,4	15% EGA	175		
GlcA	2,3,4; 2,3 or				
Glc	2,3,4,6; 2,3,6				
Ara	2,3,4; 2,3,5; 2,3; 3,5	15% BDS	175	<i>Citrus limonia</i> original and de-	1089
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4	15% EGA	175	graded gum	
Rha	2,3,4				
GlcA	2,3,4; 2,3				
(Gal	2,6; 2)				
Ara	2,3,4; 2,3,5; 2,3; 2,5	15% BDS	175	<i>Combretum leonense</i>	1090
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4	10% poly(phenyl ether)	200		
Rha	3,4; 3				
GalA	2,3				
Ara	2,3,4; 2,3,5; 2,3; 2,5; 3,5; 3	14% EGS	155	<i>Cussonia spicata</i> original and	719
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4			periodate-degraded	
GlcA	2,3,4; 2,3				
Rha	2,3,4				
(Gal	2,6; 2)				

(Continued)

TABLE XVI (Continued)

Compounds separated		Column of	Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups					
Gal	2,3,4,6; 2,3,4; 2,4,6	15% BDS	175		<i>Gum ghatti</i> periodate-degraded	1091
Man	2,3,4,6; 2,4,6; 3,4,6; 4,6	10% poly(phenyl ether)	200			
Ara	2,3,4; 2,3,5; 2,4; 2,5					
(Ara	2 Gal 2,3; 2,4; 2; Man 4)					
Ara	2,3,5	11% EGS	175		<i>Khaya ivorensis</i> original and reduced, methylated gum	1092
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4	15% BDS	175			
GlcA	2,3,4; (Glc 2,3,4)	3% XE-60	125			
GalA	2,3; (Gal 2,3)	3% ECNSS-M	175			
Rha	3,4; 3					
(Glc	3,4; Gal 2,6; 2; 3)					
Ara	2,3,5	3% ECNSS-M	175		<i>Khaya ivorensis</i> carboxyl-reduced gum	1093
Glc	2,3,4,6	15% EGA	175			
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4					
Rha	3,4; 3					
Ara	2,3,4; 2,3,5; 2,3; 2,5	15% BDS	175		<i>Khaya senegalensis</i> minor component	1094
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4	10% poly(phenyl ether)	200			
GlcA	2,3,4 or					
Glc	2,3,4; 3,4					
(Gal	2,6; 2; 4)					
Ara	2,3,5; 2,3; 2,5; 3,5	3% NPGA	150		<i>Prosopis juliflora</i> (mesquite gum)	851
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4	5% EGA	175			
Rha	2,3,4	3% XE-60	125			
GlcA	2,3,4; 2,3					
Xyl	2,3,4	3% Carbowax 6000	164		<i>Prunus avium</i> subsp. <i>avium</i> (wild cherry)	795
Ara	2,3 or 3,5; 2,5	10% poly(phenyl ether)				
Man	3,4,5					
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4					
Rha	2,3,4					
GlcA	2,3,4					
Xyl	2,3,4	5% BDS	185		<i>Prunus avium</i> var. <i>juliana</i> (cherry)	1095
Gal	2,3,4; 2,4					
GlcA	2,3,4					

Xyl	2,3,4	5% BDS	185	<i>Prunus cerasus</i> (sour cherry)	1096
Ara	3,5; 3	10% poly(phenyl ether)	200		
Gal	2,3,4; 2,4,6; 2,4				
Rha	2,3,4				
(Man	3,4,6)				
Xyl	2,3,4	3% Carbowax 6000	164	<i>Prunus domestica</i> (plum)	1097
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4	10% NPGSE	166		
Man	3,4,6				
Rha	2,3,4				
GlcA	2,3,4				
Xyl	2,3,4	5% BDS	185	<i>Prunus spinosa</i> (blackthorn)	796
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4				
Man	2,4,6				
(+ GlcA	2,3,4)				
Gal	2,3,4,6; 2,3,6	15% BDS	175	<i>Sterculia caudata</i>	1098
Rha	2,3,4; 3,4; 3	10% poly(phenyl ether)	200		
Glc(A)	2,3,4	3% NPGA	150		
GaIA	2,3,4; 2,3				
(Gal	2,6; 2; 3)				
Gal	2,3,4,6; 2,3,6	as above		<i>Sterculia setigera</i> and	1099
Rha	2,3,4; 3,4; 3			<i>Cochlospermum gossypium</i>	
Glc(A)	2,3,4				
GaIA	2,3,4; 2,3				
(Gal	2,6; 2; 3)				
Gal	2,3,4,6; 2,3,6	15% BDS	175	<i>Sterculia urens</i>	1100
Rha	2,3,4; 3,4; 3				
Glc(A)	2,3,4				
(Gal	2,3; 2,6; 2; 3)				
Glc	2,3,4,6	5% NPGA	150	<i>Sterculia urens</i> carboxyl-reduced	1040
Gal	2,3,4,6; 2,3,6; 2,6; 3,6	15% EGA	175	gum	
Rha	2,3,4; 3,4; 3				
Gal	2,3,4,6; 2,3,6; 2,4,6	15% BDS	175	<i>Ulmus fulva</i> (slippery elm)	469
Rha	2,3,4; 2,3; 3,4; 3; 4	15% EGA	175		
GaIA	2,3,4; 2,3				
Xyl	2,3,4	14% EGS	155	<i>Virgilia oroboides</i>	776
Ara	2,3,4; 2,3,5; 2,3				
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,3; 2,4				
Man	4,6				
GlcA	2,3,4; 2,3				

(Continued)

TABLE XVI (Continued)

Compounds separated			Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups	Column of				
<i>F. Mannans</i>						
Man (Gul)	2,3 2,3)	3% XE-60	175		alginic acid	88
Man	2,3,4,6; 2,4,6; 4,6	15% BDS 10% poly(phenyl ether)	175 175		<i>Phaeodactylum tricornutum</i>	1101
Man	2,3,4,6; 3,4,6; 3,4				<i>Pichia pastoris</i> and <i>Citromyces matritensis</i>	843
Man	2,3,4,6; 2,3,6; Me ₂	Apiezon M 15% BDS 10% poly(phenyl ether)	150 175 200		<i>Codium fragile</i> (green seaweed)	1102
Man	2,3,4,6; 2,3,6; 2,4,6; 2,6	15% BDS	180		<i>Torulopsis ingeniosa</i>	1103
Man	2,3,4,6; 2,3,4; 3,4,6; 3,4	5% BDS	180		<i>Candida albicans</i> (five strains)	1104
Man	2,3,4,6; 2,4,6; 3,4,6; 3,4	10% Carbowax 6000	150		<i>Torulopsis colliculosa</i>	841
Man	2,3,4,6; 2,3,6; 3,4,6; 2,3	10% Carbowax 20 M	200		<i>Mycobacterium phlei</i>	700
Man	2,3,4,6; 2,3,4; 3,4,6; 3,4	2% NPGS 2% Carbowax 20 M	175,194 150,188		<i>Mycobacterium tuberculosis</i> and <i>Mycobacterium phlei</i>	739
Man	2,3,4,6; 2,3,4; 2,4,6; 3,4,6; 3,4	15% BDS 15% poly(phenyl ether)	175 200		<i>Scarcina</i>	1105
Man	2,3,4,6; 2,3,6; 2,4,6; Me ₂	2% NPGS	143		<i>Rhodotorula glutinis</i>	1106
Man	2,3,4,6; 2,3,4; 2,4,6; 3,4,6; 3,4	10% Carbowax 6000	150		<i>Saccharomyces cerevisiae</i>	842
Man	2,3,4,6; 2,3,4; 2,4,6; 3,4,6; 3,4; 3,5	10% Carbowax 6000	168		<i>Candida albicans</i> , <i>C. parapsilosis</i> , <i>C. stellatoidea</i> , <i>C. tropicalis</i>	790
Man Xyl	2,3,4,6; 2,4,6; 2,6 2,3,4; 2,3 (MeMan)	15% BDS 15% poly(phenyl ether)	175		<i>Armillaria mellea</i>	692
Man Xyl Ara	2,3,4,6; 2,4,6 2,3,4; 2,3 2,3,4 (Man 2,6)	2% NPGS	140		<i>Trichosporon cutaneum</i>	1107

Man	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 3,4,6	0.5% NPGS	180	<i>Salmonella</i>	874
Glc	2,4,6				
Man	2,3,4,6; 2,3,5,6 (lactones)	12% Versamid		synthetic mannan	640
<i>G. Glucomannans</i>					
Glc	2,4,6; 2,4	10% NPGS	178	<i>Serratia marcescens</i> extracellular polysaccharide C	493
Man	2,4,6				
Glc	2,3,4,6; 2,3,6			extracellular polysaccharide B1	
Rha	2,4				
Glc	2,3,4,6; 2,3,4; 2,3,6 3,4,6,7-D-glycero-D-manno- heptose 2,3,4,6,7-D-glycero-D-manno- heptose 2,3,4,6,7-L-glycero-D-manno- heptose			extracellular polysaccharide B2	
Glc	2,3,4,6; 2,4,6			capsular polysaccharide B2	
Man	2,4,6				
Glc	2,3,4,6; 2,3,6			capsular polysaccharide B4	
Rha	2,4; Me				
Glc	2,3,4,6; 2,3,4; 2,3,6; 2,3 2,3,4,6,7; 3,4,6,7-D-glycero-D- manno-heptose 2,3,4,6,7-L-glycero-D-manno- heptose			capsular polysaccharide C	
Me ₄ : Me ₃ : Me ₂	ratio	15% poly(phenyl ether)	200	<i>Xanthomonas campestris</i>	756
Man	2,3,4,6; 3,4,6	3% Carbowax 20 M	150		
Glc	2,3,6; 2,6				
(Glc	2,3; 2; 3 and				
Man	2,3; 3,4)				
Glc	2,3,4,6; 2,3,6; 2,6	EG phthalate 30% methylated	210	<i>Xanthomonas hyacinthi</i> <i>X. translucens</i>	1108
Man	2,3,4,6; 3,4,6; 3,4	O-(2-hydroxyethyl)cel- lulose WP 09 (Union Carbide)		<i>X. maculofoliigardeniae</i>	

(Continued)

TABLE XVI (Continued)

Compounds separated		Column of	Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups					
Glc	2,4,6; 2,4	10% Carbowax 6000	155		<i>Serratia marcescens</i> (extra-cellular)	492
Man	2,4,6	10% NPGS	175			
Glc	2,3,4,6	10% poly(phenyl ether)	175		<i>Acetabularia crenulata</i>	699
Man	2,3,4,6; 2,3,6	7.5% BDS	175			
		10% EGA	175			
<i>H. Galactomannans</i>						
Gal	2,3,4,6	20% BDS	175		<i>Sesbania grandiflora pers</i>	392
Man	2,3,4,6					
Gal	2,3,4,6	12% EGS	150		<i>Lotus pedunculatus</i>	1109
Man	2,3,6					
(Man	2,3)					
Man	2,4,6	2% NPGS	140		<i>Candida bogoriensis</i>	663
Fuc	2,3,4; 2,4					
Gal	2,3,4,6; 3,4,6	15% BDS	185		<i>Lipomyces starkeyi</i>	1110
Man	2,3,4,6; 2,3,4; 3,4	10% Carbowax 6000	170			
Gal	2,3,5,6	10% Carbowax 6000	168		<i>Trichophyton</i>	674
Man	2,3,4,6; 2,3,4; 2,3,6; 3,4					
(Man	3,5)					
Gal	2,3,4,6	2% NPGS	134		<i>Trichosporon fermentans</i>	932
Man	2,3,4,6; 2,3,4; 3,4,6; 3,4					
Gal	2,3,5,6	10% Carbowax 6000			<i>Trichophyton granulosum</i>	1111
Man	2,3,4,6; 2,3,4; 3,4,6; 3,4	15% poly(phenyl ether)			<i>T. interdigitale</i> , <i>T. rubrum</i> , <i>T. schonleinii</i> , <i>Microsporum quinceanum</i>	
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6	15% BDS	175		soy-bean hulls	1112
(Ara	2,3; Gal Me ₂ ; Man Me ₂ ; Me)	10% poly(phenyl ether)	200			
Ara	2,3,5; 2,3; 2,5	10% NPGSE	180		honey	1113

Gal	2,3,4,6; 2,3,4; 2,4					
Man	2,3,4,6					
<i>I. Galactoglucomannans</i>						
Glc	2,3,4,6	10% Apieson N	175		<i>Picea mariana</i>	793
Gal	2,3,4,6	20% Carbowax 20 M	140-210	2	<i>Picea sitchensis</i>	
Man	2,3,4,6					
Glc	2,3,6	10% BDS	175		soybean hulls	1065
Gal	2,3,4,6	10% poly(phenyl ether)	200			
Man	2,3,4,6; 2,3,6; 2,3	3% NPGA	150			
Glc	2,3,4,6; 2,3,6				<i>Larix laricina</i> tamarack	849
Gal	2,3,4,6					
Man	2,3,4,6; 2,3,6					
(Glc	2,3; Man 2,3)					
Glc	2,3,4,6; 2,3,6	20% BDS	150		<i>Pinus maritima</i> (maritime pine)	1114
Gal	2,3,4,6; 2,3,5,6					
Man	2,3,4,6; 2,3,6					
Glc	2,3,4,6	15% BDS	180		<i>Aureobasidium</i> (<i>Pullularia</i>	677
Gal	2,3,5,6; 2,3,5; 2,3,6	15% poly(phenyl ether)			<i>pullulans</i>)	
Man	2,3,4; 2,4					
GlcA	2,3,4					
(Glc	2,3,4)					
<i>J. Polysaccharides containing fucose</i>						
Gal	2,3,4,6; 2,3,4	15% BDS	175		<i>Armillaria mellea</i>	287
Man	2,3,4,6	15% poly(phenyl ether)	200			
Fuc	2,3,4; 2,4					
(Gal	2,3; 3,4; 3; Fuc 2)					
Gal	2,3,4,6; 2,3,4	15% BDS	175		<i>Armillaria mellea</i>	692
Man	2,3,4,6; 2,3,4; 2,4,6; 3,4,6	15% poly(phenyl ether)	200			
Fuc	2,3,4; 2,4					
(Gal	3,4; 3; Man 3,4)					
Xyl	2,3,4	3% Carbowax 6000	164		<i>Fomes marginatus</i>	1115
Glc	2,3,4,6; 2,4,6					
Man	2,3,4,6; 2,4,6; 4,6					
Fuc	2,3,4					

(Continued)

TABLE XVI (Continued)

Compounds separated		Column of	Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups					
Gal	2,3,4,6; 2,3,4	15% BDS	175		<i>Polyporus pinicola</i>	332
Man	2,3,4,6	15% poly(phenyl ether)	200			
Fuc	2,3,4; 2,4					
(Gal	2,3; 3,4; Fuc 2; 3)					
Xyl	2,3,4; 3,4	15% BDS	175		<i>Polyporus pinicola</i>	332
Glc	2,3,4,6; 2,4,6	15% poly(phenyl ether)	200			
Man	2,3,4; 3,4,6; 2,6					
Fuc	2,3,4					
Xyl	2,3,4; 2,3; 3,4	5% NPGA	165		<i>Acer pseudoplatanus</i> (sycamore)	1036
Glc	2,3,4,6; 2,3,6; 3,4	15% EGA	175			
Fuc	2,3,4	10% Carbowax 20 M	185			
Xyl	2,3,4; 2,3	10% EGA	175		<i>Ascophyllum nodosum</i>	770
Fuc	2,3,4; 2,4; 3,4; 3; 2					
GlcA	2,3,4; 2,3					
<i>K. Miscellaneous polysaccharides</i>						
Ara	2,3,5	20% BDS	175		tamarind kernel	393
Xyl	2,3,4					
Glc	2,3,4,6					
Gal	2,3,4,6					
Ara	2,3,5	20% BDS	175		tamarind kernel	393
Xyl	2,3,4	20% Apieson M	150			
Glc	2,3,6; 2,3					
Gal	2,3,4,6					

Xyl	2,3,4; Me ₂	15% BDS	175	<i>Laurencia pinnatifida</i> (red alga)	412
Glc	2,3,4,6; 2,3,4; or 2,3,6; 2,4,6	15% EGA	185		
Glc	2,3,6; Me ₂	15% poly(phenyl ether)	200	<i>Arthrobacter viscosus</i>	496
Gal	2,3,6; Me ₂	3% Carbowax 20 M	145		
(ManA	2,3)				
Glc	2,3,4,6	5% NPGS	168	<i>Tolypothria tenuis</i> (blue-green alga)	363
Fru	1,3,4,6; 1,3,6				
Glc	2,3,4,6	5% NPGS	180	<i>Ophiopogon japonicus</i>	1039
Fru	1,3,4,6; 3,4,6				
Rha	2,3,4; 2,3; 2,4; 3,4	5% NPGS	135	<i>Pseudomonas aeruginosa</i>	1116
Glc	2,3,4,6; 2,4,6; 2,4	15% BDS	175	<i>Ulva lactuca</i> (green seaweed)	1117,1118
Xyl	2,3,4; 2,3; 2	10% poly(phenyl ether)	175		
Rha	2,3,4; 2,3; 2,4				
(Rha	2; 4)				
Glc	2,3,4,6; 2,4,6; 2,3	10% NPGSE	178	<i>Neisseria perflava</i>	559
Rha	3	15% poly(phenyl ether)	198		
	2,4,6,7- <i>L</i> -glycero-D-manno-heptose				
Rha	2,3,4; 2,3,5 (lactones)	10% Versamid	150	synthetic rhamnan	641
Ara	2,3,5; 2,3; 2,5; 3,5; 2; 3	15% EGA	125,150,175	<i>Brassica sinapis alba</i> (mustard-seed cotyledon)	781c
Xyl	2,3,4				
Glc	2,3,4,6; 2,3,6; 2,3				
Gal	2,3,4,6; 2,3,6; 2,4,6; 2,4				
Rha	2,3,4; 3,4; 3; 4				
Glc	2,3,4,6	7.5% BDS	175	<i>Acetabularia crenulata</i>	699
Fru	1,3,4,6; 3,4,6	10% poly(phenyl ether)	175		
		10% EGA	175		

TABLE XVII
Neutral Oligosaccharides: Structures Proved by Methylation and G.l.c.*

<i>Oligosaccharide</i>	<i>References</i>	<i>Oligosaccharide</i>	<i>References</i>
^{1 2} Araf → Ara _β	1118	^{1 4} Gal → Rha _β	1127
^{1 3} Ara → Ara _β	96,637,653,751,1081, 1085,1118a-1120	^{1 4} Gal → 2-MeGal _β	1128
^{1 3} Araf → Ara _β	637,653,1081,1085	^{1 3} 2-MeGal → Gal	1129
^{1 4} Ara → Ara _β	1121	2-MeGal → 6-MeGal	1129
^{1 5} Ara → Ara	1089,1121	^{1 4} 3-MeGal → 3-MeGal _β	1130
^{1 6} Ara → Glc	1122	^{1 4} 3-MeGal → Rha _β	1130
^{1 3} Ara → Man	1091	^{1 4} 6-MeGal → Gal _β	1129
^{1 5} Ribf → Rib _β	1123	^{1 3} 6-MeGal → 2-MeGal _β	1128
^{1 4} Xyl → Xyl _β	605,672	^{1 4} 6-MeGal → 2-MeGal _β	1128,1129
^{1 4} ^{1 4} Xyl → Xyl → Xyl _β _β	672	^{1 6} ^{1 1} Gal → Gal → Gly	1124
^{1 4} ^{1 2} Xyl → (Xyl) _n → Gly (n = 0 to 3)	646	^{1 4} ^{1 2} Gal → Gal → Thr	1040
		^{1 3} ^{1 2} Gal → Araf → Ara	105,791

$\overset{11}{\text{Gal}} \rightarrow \text{Gly}$	1124	$\overset{13}{\text{Gal}} \rightarrow \overset{13}{\text{Gal}} \rightarrow \overset{13}{\text{Gal}}$	1062,1081
$\overset{12}{\text{Gal}} \rightarrow \text{Thr}$	1040	also tetrasaccharide,	1062
$\overset{13}{\text{Gal}} \rightarrow \text{Ara}$	1085	also tri- and tetra-, as alditols	1062
$\overset{\alpha}{\text{Gal}} \rightarrow \text{Ara}$	1091,1120	$\overset{13}{\text{Gal}} \rightarrow \overset{14}{\text{Gal}} \rightarrow \overset{14}{\text{Gal}}$ (also as alditol)	1126
$\overset{\beta}{\text{Gal}} \rightarrow \text{Gal}$	653,1062,1081,1085, 1119,1121,1125, 1126	$\overset{14}{\text{Gal}} \rightarrow \overset{13}{\text{Gal}} \rightarrow \overset{13}{\text{Gal}}$ (also as alditol)	1126
$\overset{\beta}{\text{Gal}} \rightarrow \text{Gal}$ (and trisaccharide also as alditol)	1093	$\overset{16}{\text{Gal}} \rightarrow \overset{16}{\text{Gal}} \rightarrow \overset{16}{\text{Gal}}$ (also as alditol)	1062
$\overset{14}{\text{Gal}} \rightarrow \text{Gal}$	1120,1125,1126	$\overset{16}{\text{Gal}} \rightarrow \overset{16}{\text{Gal}} \rightarrow \text{Glc}$	637,1062,1081,1120
$\overset{\beta}{\text{Gal}} \rightarrow \text{Gal}$	677	$\overset{16}{\text{Gal}} \rightarrow \overset{16}{\text{Gal}} \rightarrow \text{Glc}$	1130a
$\overset{16}{\text{Gal}} \rightarrow \text{Gal}$	637,653,851,1062, 1081,1085,1120, 1121	$\overset{13}{\text{Gal}} \rightarrow \overset{13}{\text{Glc}} \rightarrow \overset{13}{\text{Glc}}$	758
as alditol	1062	$\overset{13}{\text{Gal}} \rightarrow \overset{14}{\text{Gal}} \rightarrow \overset{14}{\text{Glc}}$ (as alditol)	1131
$\overset{16}{\text{Gal}} \rightarrow \text{Glc}$	1122	$\overset{16}{\text{Gal}} \rightarrow \overset{14}{\text{Gal}} \rightarrow \overset{14}{\text{Glc}}$ (as alditol)	1131
$\overset{\beta}{\text{Gal}} \rightarrow \text{Rha}$	1093	$\overset{12}{\text{Gal}} \rightarrow \overset{12}{\text{Man}} \rightarrow \overset{12}{\text{Man}}$	675
		$\overset{16}{\text{Gal}} \rightarrow \overset{14}{\text{Man}} \rightarrow \overset{14}{\text{Man}}$	1112
		$\overset{12}{\text{Gal}} \rightarrow \overset{14}{\text{Rha}} \rightarrow \overset{14}{\text{Gal}}$ (also as alditol)	1093

(Continued)

TABLE XVII (Continued)

<i>Oligosaccharide</i>	<i>References</i>	<i>Oligosaccharide</i>	<i>References</i>
$\text{Gal} \xrightarrow[\alpha]{12} \text{Rha} \xrightarrow[\alpha]{14} \text{Gal} \xrightarrow[\alpha]{12} \text{Rha}$ (as alditol)	1093	$3\text{-MeGlc} \xrightarrow[\alpha]{14} (\text{Glc})_n$ ($n = 1,2,3$)	654
$3\text{-MeGal} \xrightarrow[\beta]{14} 3\text{-MeGal} \xrightarrow[\beta]{14} \text{Rha}$	1130	$\text{Man} \xrightarrow[\beta]{14} \text{Glc}$	1102
$3\text{-MeGal} + \text{Rha}$ tri- or tetra-saccharide	469	$\text{Man} \xrightarrow[\alpha]{12} \text{Man}$	175,838
$\text{Gal} + \text{Glc}$ tetrasaccharide	1122	$\text{Man} \xrightarrow[\alpha]{14} \text{Man}$	1112
verbascose	1130a	$\text{Man} \xrightarrow[\alpha]{14} \text{Rha}$	1135
$\text{Glc} \xrightarrow[\beta]{13} \text{Xyl}$	1117	$\text{Man} \xrightarrow[\beta]{14} \text{Rha}$	1135a
$\text{Glc} \xrightarrow[\beta]{14} \text{Glc}$	1036	$\text{Man} \xrightarrow[\alpha]{13} \text{Man} \xrightarrow[\alpha]{11} \text{Gly}$	1124
$\text{Glc} \xrightarrow[\beta]{13} \text{Man}$		$\text{Man} \xrightarrow[\alpha]{12} \text{Man} \xrightarrow[\alpha]{12} \text{Man}$ (also tetra- and penta-)	175,794a,838,842
$\text{Glc} \xrightarrow[\alpha]{14} \text{Rha}$	1132	$\text{Man} \xrightarrow[\alpha]{12} \text{Man} \xrightarrow[\alpha]{16} \text{Man}$	775
$\text{Glc} \xrightarrow[\beta]{14} \text{Rha}$	844		
$\text{Glc} \xrightarrow[\alpha\beta]{12} \text{Fru}$	479		

$\begin{array}{c} 12 \\ \text{Glc} \rightarrow \text{Glc} \\ \alpha \\ \uparrow 4 \\ \alpha \\ 1 \\ \text{Glc} \end{array}$	1133	$\begin{array}{c} 13 \quad 12 \\ \text{Man} \rightarrow \text{Man} \rightarrow (\text{also to hepta-}) \\ \alpha \quad \alpha \end{array}$	175,794a,838
$\begin{array}{c} 13 \quad 13 \\ \text{Glc} \rightarrow \text{Glc} \rightarrow \text{Glc} \\ \text{Glc}_4 \end{array}$	758,1075 1134	$\begin{array}{c} 13 \quad 13 \\ \text{Man} \rightarrow \text{Man} \rightarrow \text{Man} \\ 13 \quad 16(2) \\ \text{Man} \rightarrow \text{Man} \rightarrow \text{Man} \end{array}$	1107 1107
$\begin{array}{c} 14 \\ 4\text{-MeGlc} \rightarrow \text{Gal} \\ \alpha \end{array}$	1093	$\begin{array}{c} 14 \quad 14 \\ \text{Man} \rightarrow \text{Man} \rightarrow \text{Man} \\ \beta \quad \beta \end{array}$	769
$\begin{array}{c} 16 \\ 4\text{-MeGlc} \rightarrow \text{Gal} \\ \beta \end{array}$	767	$\begin{array}{c} 16 \quad 16 \\ \text{Man} \rightarrow \text{Man} \rightarrow \text{Man} \\ \alpha \quad \alpha \end{array}$	775
$\begin{array}{c} 16 \\ 4\text{-MeGlc} \rightarrow \text{Gal (also as alditol)} \end{array}$	1062	$\begin{array}{c} 14 \\ \text{Rha} \rightarrow \text{Gal} \\ 14 \\ \text{Rha} \rightarrow \text{Glc (also as alditol)} \end{array}$	1093 1062
$\begin{array}{c} 13 \\ 6\text{-MeGlc} \rightarrow \text{Glc} \\ \alpha \end{array}$	689	$\begin{array}{c} 14 \\ \text{Rha} \rightarrow \text{Rha} \\ \alpha \end{array}$	1062a
$\begin{array}{c} 14 \\ 6\text{-MeGlc} \rightarrow 6\text{-MeGlc and trisaccharide} \\ \alpha \end{array}$	689	$\begin{array}{c} 14 \quad 16 \\ \text{Rha} \rightarrow \text{Glc} \rightarrow \text{Gal (also as alditol)} \\ \alpha \end{array}$	1062
		$\text{Rha} \rightarrow \text{Xyl} \rightarrow \text{Glc}$	1117

^a All sugars pyranose, except as noted; all sugars of D configuration, except L-arabinose and L-rhamnose; Gly = glycerol; Thr = threitol.

$\text{GlcA} \xrightarrow{13} \text{Man}$	R	492,493,775,1101	$4\text{-MeGlcA} \xrightarrow{12} \text{Xyl} \xrightarrow{12} \text{Xyl}$		1137
$\text{GlcA} \xrightarrow{14} \text{Rha}$	R	1117,1118	$4\text{-MeGlcA} \xrightarrow{\alpha} \text{Xyl} \xrightarrow{\beta} \text{Xyl}$	R,A	1141
$\text{GlcA} \xrightarrow{12} \text{GlcA}$		781a	$4\text{-MeGlcA} \xrightarrow{\alpha} \text{Xyl} \xrightarrow{\beta} \text{Xyl}$	R	1144
$\text{GlcA} \xrightarrow{12} \text{Xyl} \xrightarrow{14} \text{Xyl}$	R,A	1141	$\text{Xyl} \xrightarrow{14} \text{Xyl}$	R	1144
$\text{GlcA} \xrightarrow{16} \text{Glc} \xrightarrow{14} \text{Glc}$			$\text{4-MeGlcA} \xrightarrow{\alpha} \text{Gal} \xrightarrow{13} \text{Gal}$		851
$\text{Glc} \xrightarrow{14} \text{Glc}$			<i>C. Galacturonic acid</i>		
$\text{Glc} \xrightarrow{14} \text{Glc}$			$\text{Xyl} \xrightarrow{13} \text{GalA}$	A	1070,1136
$\text{Glc} \xrightarrow{14} \text{Glc}$			$\text{Ara} \xrightarrow{13} \text{GalA}$	A	1136
$\text{Glc} \xrightarrow{14} \text{Glc}$			$\text{GalA} \xrightarrow{14} \text{Gal}$		1093,1138a,1145
$\text{Glc} \xrightarrow{14} \text{Glc}$				also R	1092,1100
$\text{Glc} \xrightarrow{14} \text{Glc}$			(note correction to anomeric linkage)		1145
$\text{Glc} \xrightarrow{14} \text{Glc}$			$\text{GalA} \xrightarrow{16} \text{Gal}$		751
$\text{GlcA} \xrightarrow{14} \text{Glc} \xrightarrow{13} \text{Man}$		1047a	$\text{GalA} \xrightarrow{12} \text{Rha}$		1036,1092,1136,1137, 1145
$\text{GlcA} \xrightarrow{12} \text{Man} \xrightarrow{12} \text{Man}$		871		A	1145
$\text{GlcA} \xrightarrow{13} \text{Man} \xrightarrow{12} \text{Man}$	R	1101			
$\text{GlcA} \xrightarrow{14} \text{Rha} \xrightarrow{13} \text{GlcA} \xrightarrow{13} \text{Xyl}$	R	1117,1118			

(Continued)

TABLE XVIII (Continued)

Oligosaccharide ^a		References		Oligosaccharide ^b		References	
$\text{GalA} \xrightarrow{12} \text{Rha} \xrightarrow{14} \text{Gal}$	R	318		$\text{GalA} \xrightarrow{14} \text{GalA} \xrightarrow{12} \text{Rha}$	R	1136 1092,1093	
$\text{GalA} \rightarrow \text{Rha} \rightarrow \text{Gal}$	A	1145 1145		$\text{GalA} \xrightarrow{12} \text{Rha} \xrightarrow{14} \text{GalA}$	A R/A	1070 1145	
$\text{Gal} \xrightarrow{12} \text{GalA} \xrightarrow{14} \text{Gal}$		1145		$\text{Gal} \xrightarrow{12} \text{Rha} \xrightarrow{14} \text{GalA} \xrightarrow{14} \text{Rha}$	A	1070,1092	
$\text{GalA} \xrightarrow{12} \text{Rha} \xrightarrow{12} \text{Rha}$	A	1136 1070		$\text{GalA} \rightarrow \text{Rha} \rightarrow \text{GalA} \rightarrow \text{Rha}$	A	1136 1136	
$\text{Gal} \xrightarrow{13(4)} \text{GalA} \xrightarrow{12} \text{Rha}$	R	1120		<i>D. Glucuronic and galacturonic acids</i>			
$\text{GalA} \xrightarrow{14} \text{GalA}$	R	1069,1070,1092,1136, 1137		$\text{GlcA} \xrightarrow{13} \text{GalA}$	R	1092,1093,1145	
$\text{GalA} \xrightarrow{14} \text{GalA} \xrightarrow{14} \text{Gal}$	R	1070,1136		$\text{GlcA} \xrightarrow{13} \text{GalA} \xrightarrow{12} \text{Rha}$	R	1100 1092,1093,1100,1145	

^a All sugars pyranose and all of D configuration, except for L-arabinose, L-fucose, and L-rhamnose.

^b R signifies that the acidic compound was investigated as the neutral derivative, that is, $-\text{CO}_2\text{H} \rightarrow -\text{CH}_2\text{OH}$. A signifies that the reducing group was converted into a hydroxymethyl group; that is, $-\text{CHO} \rightarrow -\text{CH}_2\text{OH}$.

TABLE XIX

Basic Oligosaccharides: Structures Proved by Methylation and G.l.c.

<i>Oligosaccharide</i>	<i>References</i>
$\begin{array}{c} \text{GalNAc} \xrightarrow[13]{\alpha} \text{Gal} \end{array}$	716
$\text{GlcNAc} \xrightarrow[14]{\alpha} \text{Gal}$	716
$\text{GlcNAc} \xrightarrow[16]{\beta} \text{Gal}$	716
$\text{GlcNAc} \xrightarrow[14]{\alpha} \text{Gal} \xrightarrow[13]{\beta} \text{GalNAc}$	716
$\text{GlcNAc} \xrightarrow[14]{\alpha} \text{Gal} \xrightarrow[14]{\beta} \text{GlcNAc}$	716
$\text{Gal} \xrightarrow[14]{\beta} \text{GlcNAc} \xrightarrow[16]{\beta} \text{GalNAc}$	1146
$\text{Gal} \xrightarrow[14]{\beta} \text{GlcNAc} \xrightarrow[13]{\beta} \text{Gal}$	1146
$\begin{array}{c} \uparrow_3 \\ \text{Fuc} \end{array}$	

TABLE XX
Trimethylsilyl Ethers of Methyl O-Methylglycosides

<i>Compounds separated</i>			<i>Column temp.</i> (°C)	<i>Rate</i> (deg. min ⁻¹)	<i>Material or problem studied</i>	<i>References</i>
<i>Sugar residue</i>	<i>Position of methyl ether groups</i>	<i>Column of</i>				
Ara	2,3,4; 3,4	10% BDS	175		model	379
Ara	2,3,5	5% SE-52	140-155	1	<i>Picea abies</i> (Norway spruce)	601
Xyl	2,3,4; 2,3; 2; 3				<i>Tilia americana</i> (basswood)	846
					<i>Larix laricina</i> (tamarack)	848
Ara	2,3,4; 2,3,5; 2,5	5% SE-52	140-155	1	<i>Larix laricina</i> (tamarack)	850
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4					
Glc	2,3,4,6				scillabiose synthesis	844
Rha	2,3					
Glc	2; 3; 4; 6	19.5% Carbowax 20 M	150		hepta- <i>O</i> -acetylmaltose	402
Glc	2,3; 2,6; 2; 3; 4; 6 (ethyl ethers)	7% Carbowax 20 M			vinylation	665
Glc	2,3,4,6; 2,3,6; 2,3	5% SE-52	140-155	1	<i>Larix laricina</i> (tamarack)	849
Gal	2,3,4,6					
Man	2,3,4,6; 2,3,6; 2,3					

Gal	2,3,4,6; 2,4,6	10% BDS	175		model	379
Gal	2,3,4,6	20% BDS	175		<i>Sesbania grandiflora</i>	392
Man	2,3,6; 2,3					
Gal	2,3,4	3% QF-1	140		α -D-galactopyranosyluronic acid-L-	845
Rha	3,4				rhamnopyranose 1,2';1',2-	
					dianhydride	
Man	2,3,4,6; 2,3,4; 3,4,6; 3,4	2% NPGS	134		<i>Trichosporon fermentans</i>	547,675,843
Man	2,3,4,6; 2,4,6; 3,4,6; 3,4	10% Carbowax 6000	115		<i>Torulopsis colliculosa</i>	841
Man	2,3,4,6; 2,3,4; 2,4,6;	10% Carbowax 6000	135		<i>Saccharomyces cerevisiae</i>	842
	3,4,6; 3,4					
		2.3% NPGS	140		<i>Saccharomyces cerevisiae</i>	175,794a,838
Man	2,3,4,6; 2,3,4; 2,4,6;	10% Carbowax 6000	115		<i>Candida albicans</i> , etc.	790
	3,4,6; 3,4; 3,5					
Man	monomethyl	3% SE-52	60-150	1	methylation of Me α -D-mannoside	782
		4% SE-52	60-165	1		
Man	Me ₄ ; Me ₃ ; Me ₂	2% NPGS	136		model	824
GlcNHMe		10% NPGSE	140		model	827
GalNHMe					yeast mannan	830

TABLE XXI
Acetates of Methyl O-Methylglycosides

<i>Compounds separated</i>		<i>Column of</i>	<i>Column temp. (°C)</i>	<i>Material or problem studied</i>	<i>References</i>
<i>Sugar residue</i>	<i>Position of methyl ether groups</i>				
Ara	2,3,4; 2,3,5; 2,5	15% EGS		<i>Larix laricina</i> (tamarack)	850
Gal	2,3,4,6; 2,3,4; 2,4,6; 2,4				
Glc	2,3,6	15% LAC 4R-886	170	<i>Acer saccharum</i> (sugar maple)	673
Glc	2,3; 2,4; 2,6; 4,6	15% EGS		<i>Poria cocos</i>	797
				<i>Larix laricina</i> (tamarack)	847
Glc	2,3,4,6; 2,3,4; 2,3,6; 2,3; 2	5 or 10% LAC 4R-886	180,195	enzymic deacylation	383
Glc	Various	15% LAC 4R-886	190	model	816
Gal	2,3,4; 2,3,6; 2,4,6; 2,4	3% XE-60	125	<i>Prosopis juliflora</i> (mesquite gum)	851
Gul	2,3,4; 2,3	3% XE-60	150,175,200	alginic acid	88
Man	2,3,4; 2,3				

TABLE XXII
Trimethylsilyl Derivatives of *O*-Methyl Sugars

<i>Compounds separated</i>		<i>Column of</i>	<i>Column temp. (°C)</i>	<i>Rate (deg. min⁻¹)</i>	<i>Material or problem studied</i>	<i>References</i>
<i>Sugar residue</i>	<i>Position of methyl ether groups</i>					
Xyl	2	3% JXR	140 to 210	2 min 3	model	407
Xyl	2,3,4; 2,3; 2; 3; 4	3% SE-52 15% EGA	50-125	5.6	model	852
Ara Xyl	2,3,5 2,3,4; 2,3; 2; 3	5% SE-52	130-160	1	<i>Picea abies</i> (Norway spruce)	601
Ara Xyl	2,3,5 2,3,4; 2,3; 2; 3	8% SE-52	110-140	3	<i>Zea mays</i>	701
Glc	3	3% OV-1	170		mass spectrum	200
Glc	3	3% SE-52			aldobiouronic acid kinetics	295
Glc	4	5% SE-30	160		4- <i>O</i> -methylmalto-oligosaccharides	296
Glc	6	5% SE-30	140	2	6- <i>O</i> -methylamylose	856
Glc	4Me; 4Et; 4Bu	5% SE-30	160		4- <i>O</i> -butylmalto-oligosaccharides	651
Glc	2; 3	3% SE-52			substitution at O-2 or O-3 of glucose	853
Glc	2,3; 2; 3				cellulose model	1147

(Continued)

TABLE XXII (Continued)

<i>Compounds separated</i>			<i>Column temp. (°C)</i>	<i>Rate (deg. min⁻¹)</i>	<i>Material or problem studied</i>	<i>References</i>
<i>Sugar residue</i>	<i>Position of methyl ether groups</i>	<i>Column of</i>				
Glc	2; 3; 4	5% BDS	90-170	2	pustulan	854
Glc	3,4,6; 3,4; 4,6				synthesis	821,822
Glc	2; 3; 4; 6	19.5% Carbowax 20 M	150		hepta- <i>O</i> -acetylmaltose	402
Glc	2; 3; 4; 6	10% Carbowax	170		<i>Mycobacterium phlei</i>	654
Glc	2,3,4,6; 2,3,6; 2,3; 3	6% SE-52	100-180	15	cellulose	794
Glc	2,3; 2,4; 3,4; 2; 3; 4; 6	5% BDS	90-170 or 100	2	dextran	855
Glc	2,3,4,6; 2,3,6; 2,3; 2,6; 3,6; 4,6; 2; 3; 6	0.2% poly(1,10-decane- diol succinate)	125		model for cellulose (first three liquid phases on glass beads)	819,857
		0.2% hexatriacontane	125			
		0.2% SE-30	125			
		8.7% EGS	125			

Glc	2,3,4,6	poly(ethylene glycol)	130	synthesis of trehalose and analogs	859
Gal	2,3,4,6	1500 K			
Glc	2,3,4,6; 2,4,6	3% ECNSS-M		<i>Klebsiella</i> K24	1052
Man	2,3,4,6; 3,4,6	BDS SE-52			
Gal	3	15% BDS	175	<i>Armillaria mellea</i>	333
Gal	2,3,4,6; 2,3,6; 2,3	5% SE-52 10% EGS		<i>Larix laricina</i> (tamarack)	858
Gal	3,4	3% QF-1	140	α -D-galactopyranosyluronic acid- L-rhamnopyranose 1,2';1',2- dianhydride	845
Rha	3,4				
Gal	2,3,4,6	20% NPGS	180	<i>Caesalpinia pulcherrima</i>	1148
Man	2,3,4,6; 2,3,6; 2,4,6; 3,4,6; 2,3				
Man	2,6	10% NPGSE	200	synthetic	1058
Man	2,3,4,6; 2,3,6; 3,4,6; 2,3	10% Carbowax 20 M	140	<i>Mycobacterium phlei</i>	700
Man	2,3,4,6; 2,3,5,6; 2,5,6; 3,5,6	2% NPGS	136	model	824

TABLE XXIII
Acetates and Trifluoroacetates of O-Methyl Sugars

Compounds separated		Column of	Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups					
A. Acetates						
Glc	tetra-,tri-,di-, and mono-ethers				model	817
Glc Rha	2,3,4,6 2,3	5% BDS	110-210	2	synthesis of scillabiose	826b,844
Glc Man	2,4,6 2,3,4,6; 3,4,6	3% ECNSS-M	170		<i>Klebsiella</i> K24	1052
Glc Man	2,3,4,6; 2,3,6; 2,3 2,3,4,6	3% ECNSS-M	185		model glucomannans <i>Picea mariana</i> and <i>P. sitchensis</i>	826b 793
Glc Gal Man	2,3,4,6 2,3,4,6; 2,3,5,6 2,3,4,6	3% ECNSS-M	170		model glucomannans <i>Picea mariana</i> and <i>P. sitchensis</i>	826b 793
B. Trifluoroacetates						
Glc	2; 3; 4; 6	1% XE-60	130-150 120 then to 150	1 4 min 2	model	223

TABLE XXIV
Methylated Aldonolactones

<i>Compounds separated</i>		<i>Column of</i>	<i>Column temp. (°C)</i>	<i>Material or problem studied</i>	<i>References</i>
<i>Sugar residue</i>	<i>Position of methyl ether groups</i>				
Ara	2,3,5; 2,5; 3,5	5% NPGA	150	<i>Araucaria bidwilli</i> gum	1062
Ara	2,3,5	7% Versamid	155	<i>Centrosema plumari</i>	869
Gal	2,3,4,6				
Ara	2,3,5; 2,3; 2,5; 3,4; 3,5	5% EGA	175	mesquite gum	803,851
Gal	2,3,4,6; 2,3,6				
Ara	2,3,5; 2,3	15% EGA	175	<i>Acer pseudoplatanus</i> (sycamore)	1036
Gal	2,3,4,6				
Xyl	2,3,4; 2,3; 2,4				1036
Glc	2,3,4,6; 2,3,6				
Fuc	2,3,4				
Gal	2,3,4,6; 2,3,5,6; 2,3,5	12% Versamid		synthetic galactan	207
Man	2,6	10% NPGSE	200	synthesis	1058
Man	2,3,4,6; 2,3,5,6	12% Versamid		synthetic mannan	640
Rha	2,3,4; 2,3,5	10% Versamid	150	synthetic rhamnan	641

TABLE XXV
Trimethylsilyl Ethers of Methylated Alditols

<i>Compounds separated</i>		<i>Column of</i>	<i>Column temp. (°C)</i>	<i>Material or problem studied</i>	<i>References</i>
<i>Sugar residue</i>	<i>Position of methyl ether groups</i>				
Glc	3	19.5% Carbowax 20 M	150-160	hepta- <i>O</i> -acetylmaltose	402
Glc	2,3,4,6; 2,3,6; 2,4,6; 3,4,6	2% OV-1 2% FX-1105	140 160	<i>Clerodendron trichotomum</i> (flavanoid)	781a
Glc	2,3,4,6; 2,3,6; 2,4,6; 2,3; Me ₂	30% Apiezon L 3% SE-30	190 130	<i>Stereocaulon paschale</i> (a lichen)	860
Glc	2,3,4,6; 2,3,6; 2,3; 2,6; 3,6; 2; 3; 6	2% SE-52	175	reaction of cellulose with formaldehyde	820

TABLE XXVI
Acetylated, Methylated Alditols

Compounds separated			Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups	Column of				
A. Pentosans						
Ara	2,3,4; 2,3,5; 2,3; 3,4; 3,5; 2; 3; 4; 5	15% LAC 4R-886	200		model	798
Ara	2,3,5	3% XE-60			wheat pentosan	646
Xyl	2,3; 2					
Ara	2,3,5	3% ECNSS-M	150		<i>Pinus radiata</i>	1149
Xyl	2,3,4; 2,3; 2; 3					
Ara	2,3,5	3% ECNSS-M	170	10 min	<i>Thuja plicata</i> (western red	762
Xyl	2,3,4; 2,3; 2; 3		to 220	2	cedar)	
Ara	2,3,4; 2,3,5; 2,3; 2; 3	3% XE-60	170		soybean-cotyledon meal	1031a
Gal	2,3,6; 2,6					
Ara	2,3	3% ECNSS-M	180		<i>Solanum tuberosum</i> (potato)	755
Gal	2,3,4,6; 2,3,6; 2,3; 2,6					
Ara	2,3,5; 2,3; 3,5	3% ECNSS-M			<i>Arena sativa</i> (oat; galactoarabinoxylan)	1035
Xyl	2,3,4; 2,3; 2; 3					
Gal	2,3,4,6					
Ara	2,3,5	3% ECNSS-M			<i>Arena sativa</i> (oat, delignification)	1034
Xyl	2,3,4; 2,3; 2					
Glc	2,3,4,6; 2,3,6; 2,4,6					
Gal	2,3,4,6					
Xyl	2,3,4; 2,3; 3	3% ECNSS-M	160		<i>Eucalyptus globulus</i> <i>Betula verrucosa</i>	1032

(Continued)

TABLE XXVI (Continued)

Compounds separated			Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups	Column of				
Xyl	2,3,4; 2,3; 3	3% ECNSS-M	150		<i>Bombax malabaricum</i> (red cotton-wood)	685
Xyl	2,3,4; 2,3; 2; 3	3% ECNSS-M	160 to 180	3 min 2	<i>Zea mays</i>	701
Xyl Glc	2,3,4; 2,3; 3 2,3,4	3% ECNSS-M 3% OV-225	170		<i>Cordyline indivisa</i>	1033
<i>B. Glucans</i>						
Glc	3	10% ECNSS-M (and other columns)	170-220	0.8	soil samples	91
Glc	2,3,4 (2,3,4,6; 2,4,6; 2,4)	3% ECNSS-M	180		<i>Aureobasidium pullulans</i>	1078
Glc	2,3,6	10% LAC 4R-886 (equiv. to EGS)	170		sugar-maple roots	673
Glc	1,2,3,4,5	10% NPGS	168		synthetic arabinan	1030
Glc	2,3,4,6; 2,3,4; 2,4	3% ECNSS-M	180		dextran	749
					<i>Stereum sanguinolentum</i>	763
Glc	2,3,4,6; 2,3,4; 2,4,6	3% ECNSS-M	180		pustulan	764
Glc	2,3,4,6; 2,3,4; 2,4 (mixed ethers; Me and Et)	3% ECNSS-M	170		dextran	765
Glc	2,3,4,6-Pr ₄	13% EGS	200		<i>Mycobacterium phlei</i>	654
Glc	6-Me-2,3,4-Pr ₃					
Glc	3-Me-2,4,6-Pr ₃					

Glc	2,3,4,6; 2,3,6; 2,3; 2,6	0.2% EGA 0.2% EGS 0.4% XF-1150	150	<i>Rhodymenia pertusa</i> (red seaweed)	865,866
Glc	2,3,4,6; 2,3,6; 2,4,6; 3,4,6	2% XF-1105 2% OV-1	180 160	<i>Clerodentron trichotomum</i> (flavanoid)	781a
Glc	2,3,4,6; 2,3,4; 2,3,6; 2,4,6	3% ECNSS-M	180	<i>Fusicoccum amygdali</i>	718
Glc	2,3,4,6; 2,3,4; 2,4,6; 2,4	3% ECNSS-M		<i>Fomes annosus</i>	781b,922
Glc	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4	3% ECNSS-M	180	<i>Basidiomycete</i> QM 806	757
Glc	2,3,4; 2,3; 2,4; 3,4; 2; 3; 4	3% ECNSS-M	192	partially acetylated dextran	1150
Glc	2,3,6; 2,3; 2,6; 3,6; 2; 3; 6	1% polyester (LAC IR-296)	210	cellulose acetate	87
Glc	2,3,4,6; 2,3,6; 2,3; 2,6; 3,6; 2; 3; 6	3% EGS (LAC 4R-866)	220	reaction of cellulose with formaldehyde	820
<i>C. Galactans</i>					
Gal	6	5% Apiezon M + 1% poly(ethylene glycol)	200	separation from β -D-Gal pentaacetate	864
Gal	1,2,3,5,6	5% NPGS	167	<i>Panax ginseng</i> pectin <i>Zosteraceae</i> pectin	104
Gal	2,6	20% Apiezon M	220	<i>Laurencia pinnatifida</i> (red alga)	412
Gal	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,3; 2,4; 2,6; 4,6; 2; 4; 6	3% ECNSS-M	175	model	834
Glc	2,3,4,6; 2,3,4	3% ECNSS-M	180	<i>Fusicoccum amygdali</i>	718
Gal	2,3,4,6; Me ₈ ; 2,6				

(Continued)

TABLE XXVI (Continued)

Compounds separated			Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups	Column of				
Glc	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,3	3% ECNSS-M OS-138 (SCOT)	170 190		<i>Rhizobium meliloti</i>	445
Gal	2,3,4,6; 2,4,6					
<i>D. Mannans</i>						
Man	1,2,3,4,5				laminaran	1076
Man	2,3,4,6; 2,3,4; 2,4,6; 3,4,6; 2,4; 3,4	3% ECNSS-M	175		<i>Polyporus borealis</i>	678
Glc	2,3,4,6; 2,3,6	3% ECNSS-M	165		<i>Stylosanthes humilis</i>	1041
Gal	2,3,4,6					
Man	2,3,6; 2,3					
Glc	2,3,4,6	3% ECNSS-M	180		<i>Aureobasidium pullulans</i>	677
Gal	2,3,5,6; 2,3,5; 2,3,6	15% poly(phenyl ether)	200			
Man	2,3,4; 2,4					
Glc	2,3,4,6; 2,3,6; 2,3	3% ECNSS-M	170		<i>Cordyline indivisa</i>	1033
Gal	2,3,4,6	3% ECNSS-M	170	10 min	<i>Picea sitchensis</i> and	793
Man	2,3,4,6; 2,3,6; 2,3		to 190	2	<i>Picea mariana</i>	
		3% ECNSS-M	170	10 min	<i>Thuja plicata</i>	762
			to 220	2		
<i>E. Capsular polysaccharides</i>						
Glc	2,3,6; 2,3	3% ECNSS-M	180		<i>Klebsiella</i> K5	1047,1047a
Man	2,4,6; 2					
Glc	2,4,6; 3,4; 3	3% ECNSS-M	180-220	2	<i>Klebsiella</i> K24	1052
Man	2,3,4,6; 2,4,6; 3,4,6	5% BDS 15% OS-138				

Glc	2,3,4,6; 2,4,6	20% Apiezon L	218		<i>Klebsiella</i> K72	1046
Rha	2,4; 3,4					
Glc	2,3,4,6; 2,3,4; 2,3,6	3% ECNSS-M	165		<i>Pneumococcus</i> II	484a
Rha	2,3,4; 2,4; 3,4	OV-225 (SCOT)				
Glc	2,3,4	3% ECNSS-M	180-220	2	<i>Klebsiella</i> K20	1048,1049
Gal	2,3,4,6; 2,4,6					
Man	2,3,4,6; 3,4,6; 4,6					
Glc	2,4; 2,6; 2	3% ECNSS-M	180		<i>Klebsiella</i> K21	861,862
Gal	2,3,4,6; 2,4,6; 2,3	5% BDS	180-200	2		
Man	2,3,4,6; 2,4,6; 3,4,6					
Glc	2	3% ECNSS-M	150-220	2	<i>Klebsiella</i> K56	1053
Gal	2,4,6; 4,6					
Rha	2,3,4					
Glc	2,3,4	3% OV-225	170		<i>Klebsiella</i> K9	720
Gal	2,4,6	3% ECNSS-M	170			
Rha	2,4; 3,4; 2					
Gal	2,4,6	15% OS-138	240		<i>Klebsiella</i> K32	1054
Rha	2,3; 2,4					
<i>F. Algal and fungal polysaccharides</i>						
Ara	2,4	3% XE-60	150-200		alginic acid	88
Gul	2,3,4; 2,3; 1,6-anhydro-2,3					
Man	2,3,4; 2,3					
Xyl	2,3,4; 2,3	3% ECNSS-M	162		<i>Ascophyllum nodosum</i>	770
Fuc	2,3,4; 2,4; 3,4; 2; 3					
Xyl	3,4	3% ECNSS-M	175		<i>Fomes annosus</i>	922
Man	2,4,6; 2,6				<i>Polyporus pinicola</i>	463
Fuc	2,3,4					

(Continued)

TABLE XXVI (Continued)

Compounds separated			Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups	Column of				
Gal Fuc	2,3,4,6; 2,3,4; 2,4 2,3,4	3% ECNSS-M	175		<i>Polyporus borealis</i>	678
Gal Fuc	2,3,4,6; 2,3,4; 3,4 2,3,4	3% ECNSS-M			<i>Fomes annosus</i> <i>Polyporus ovinus</i>	922 464
Gal Man Fuc	2,3,4,6; 2,3,4; 3,4,6; 3,4 2,3,4,6; 2,3,4 2,3,4; 2,4 (2 unknowns)	3% ECNSS-M	180		<i>Polyporus fomentarius</i> <i>Polyporus igniarius</i>	462
<i>G. Gums</i>						
Ara Gal	2,3,4; 2,3,5; 2,3; 2,5; 3,5; 3 2,3,4; 2,3,6; 2,4,6; 2,4; (2,6; 2)	3% ECNSS-M	175		<i>Cussonia spicata</i>	719
Ara Glc Gal Rha	2,3,4; 2,3,5; 2,3; 2,5; 3,5 2,3,4; 2,3 2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 2,4 2,3,4	5% BDS	140-205	3	<i>Citrus limonia</i> (lemon gum)	863
<i>H. Lipopolysaccharides</i>						
Glc Gal Fuc	2,3,4,6; 2,4,6 2,3,4,6; 2,4,6; 2,6 2,3,4; 2,3; 2	3% ECNSS-M			M antigen	883
Glc Gal Fuc	2,4,6; 2,3 2,4,6; 2,3; 2,6 2,3; 2	3% ECNSS-M			M antigen	443

Glc	2,3,4,6; 2,4,6; 2,3	3% ECNSS-M	150	M antigen	444
Gal	2,3,4,6; 2,4,6; 2,6				
Fuc	2,3,4; 2,3; 2				
Gal	2,3,4,6; 2,4,6	3% ECNSS-M		<i>Klebsiella</i> O groups 1 and 6	1044
Rib	3,5	3% ECNSS-M	170	<i>Klebsiella</i> O group 4	712
Gal	2,3,4,6; 2,3,6				
Glc	2,3,4,6	3% ECNS-M		<i>Klebsiella</i> O group 5	787
Man	2,3,4,6; 2,4,6; 3,4,6			(3-O-methyl-D-mannose)	
Rib	2,5	OS-138 (SCOT)		<i>Klebsiella</i> O group 10	432
Glc	2,3,4,6			(3-O-methyl-L-rhamnose)	
Rha	2,3,4; 2,3; 2,4				
Glc	2,3,4,6	3% ECNSS-M		<i>Klebsiella</i> O group 9	713
Gal	1,2,4,5,6; 2,3,4,6; 2,3,5,6; 2,4,6; 2,5,6; 4,6	OV-225 (SCOT)			
Glc	2,3,4,6	3% ECNSS-M	170	<i>Salmonella newport</i>	727
Gal	2,4,6	3% OV-225	170	<i>Salmonella kentucky</i>	
Man	2,3,4,6; 3,4,6; 4,6				
Rha	2				
Glc	2,3,4,6	3% ECNSS-M	50 up	<i>Salmonella newport</i>	435
Gal	2,4,6	OV-225 (SCOT)	200		
Man	2,3,4,6; 3,4,6; 2,4,6				
Rha	1,2,3,5; 2,3,4				
Glc	2,3,4,6	3% ECNSS-M	155,170	<i>Salmonella typhi</i>	681
Gal	2,3,4,6; 2,3,6; 2,4,6; 2,6	15% OS-138	180	<i>Salmonella enteritidis</i>	
Man	3,4,6				
Rha	2,3				
Tyv	2,4				

(Continued)

TABLE XXVI (Continued)

<i>Compounds separated</i>			<i>Column temp. (°C)</i>	<i>Rate (deg. min⁻¹)</i>	<i>Material or problem studied</i>	<i>References</i>
<i>Sugar residue</i>	<i>Position of methyl ether groups</i>	<i>Column of</i>				
Glc	2,3,4,6; 3,4,6; 2,4; 3,6	3% ECNSS-M	150		<i>Salmonella typhimurium</i>	832
Gal	2,3,4,6; 2,4,6; 3,4,6; 2,6				(correction of Refs. 16, 440a; model study of Me ₆ -Glc)	
Glc	2,3,4,6; 2,4; 3,6	3% ECNSS-M			<i>Salmonella muenster</i>	437
Gal	2,3,4,6; 2,4,6; 3,4,6; 2,6	OV-225 (SCOT)			<i>Salmonella newington</i>	440
Man	2,3,4,6; 2,3,4					
Rha	2,3					
Glc	2,3,4,6; 2,4	3% ECNSS-M	170		<i>Salmonella</i>	16
Gal	2,3,4,6; 2,4,6; 2,6	15% OS-138	180			
Man	2,4,6; 4,6					
Rha	2,3					
Abe	2,4 (3,6-hexose)					
Glc	2,3,4,6; 2,4	3% ECNSS-M	170		<i>Salmonella typhimurium</i>	440a
Gal	2,3,4,6; 2,4,6; 2,6; 3,6	OS-138			LT2 (see Ref. 832 for correction)	
Man	2,4,6; 4,6				<i>Salmonella typhimurium</i> SR	1042
Rha	2,3					
Abe	2,4 (3,4,6-hexose)					

Glc	2,3,4,6; 3,4,6; 2,4	3% ECNSS-M		<i>Salmonella strasbourg</i>	1045
Gal	2,3,4,6; 2,4,6; 3,4,6; 2,6; 3,6				
Man	2,3,4; 2,4,6; 2,4				
Rha	2,3				
Tyv	2,4				
Glc	2,3,4,6; 2,3,4; 2,4; 3,6	3% ECNSS-M	170	<i>Salmonella paratyphi A</i>	439
Gal	2,3,4,6; 2,4,6; 3,4,6; 2,6	OV-225 (SCOT)		var. durazzo	
Man	2,3,4,6; 2,4,6; 4,6				
Rha	2,3				
Par	2,4				
Glc	2,3,4,6; 3,4,6; 2,4; 3,6	3% ECNSS-M		<i>Escherichia coli</i> 0100 core	729
Gal	2,3,4,6; 2,3,6; 2,4,6				
Man	2,4,6				
Rha	2,3				
Abe	2,4				
Hept	2,3,4,6,7; 2,4,6,7; 2,4,6				
Glc	2,3,4,6; 3,4,6; 2,4; 3,6	3% ECNSS-M		<i>Salmonella senftenburg</i>	438
Gal	2,3,4,6; 2,3,4; 2,4,6; 3,4,6; 2,4; 2,6	OV-225 (SCOT)			
Man	2,3,4,6; 2,3,4				
Rha	2,3				
Glc	2,3,4,6; 2,4,6; 2,4; 3,6; 2; 4	3% ECNSS-M		<i>Salmonella</i> hybrids B/D	730
Gal	2,3,4,6; 2,4,6; 3,4,6; 2,4; 2,6; 4,6				
Man	2,4,6; 4,6				
Rha	2,3				
Abe	2,4; 2				
Tyv	2,4				

(Continued)

TABLE XXVI (Continued)

Compounds separated		Column of	Column temp. (°C)	Rate (deg. min ⁻¹)	Material or problem studied	References
Sugar residue	Position of methyl ether groups					
Glc	2,3,4,6; 2,4; 3,6; 2	3% ECNSS-M			<i>Salmonella</i> hybrid B/C ₂	728
Gal	2,3,4,6; 2,4,6; 3,4,6; 4					
Man	2,4,6					
Rha	2,3; 2; 3					
Abe	2,4					
Hept	2,3,4,6,7; 6					
GlcNMe	3,4,6					
<i>I. Miscellaneous</i>						
Xyl	3	3% ECNSS-M	155-165		<i>Myxococcus fulvus</i> <i>Rhodopseudomonas viridis</i>	711
Man	2,4; 3,6	3% ECNSS-M			synthesis	825
Abe	2; 4	3% ECNSS-M	140		synthesis	833
Pentoses and hexoses		3% ECNSS-M	180,200,220		model	831
GlcN	3,4,6; 3,4	10% NPGSE	245		<i>Serratia marcescens</i> lipid A	860a

TABLE XXVII
Trifluoroacetates of Methylated Alditols

<i>Compounds separated</i>		<i>Column of</i>	<i>Column temp. (°C)</i>	<i>Rate (deg. min⁻¹)</i>	<i>Material or problem studied</i>	<i>References</i>
<i>Sugar residue</i>	<i>Position of methyl ether groups</i>					
Glc	2; 3; 4; 6	1% XE-60	130-150	1	model	223
Glc	2,3,4,6; 2,3,6; 2,4,6; 3,4,6	2% OV-1	110		<i>Clerodendron trichotomum</i> (flavanoid)	781a
Glc	2,3,4,6; 2,3,6; 2,3; 2,6; 3,6; 2; 3; 6	3% SE-52	125		reaction of cellulose with formaldehyde	820

TABLE XXVIII
Methylated Aldononitrile Acetates

<i>Compounds separated</i>		<i>Column of</i>	<i>Column temp. (°C)</i>	<i>Rate (deg. min⁻¹)</i>	<i>Material or problem studied</i>	<i>References</i>
<i>Sugar residue</i>	<i>Position of methyl ether groups</i>					
Xyl	2; 3	3% ECNSS-M	180,200		peat	867
Gal	3	3% ECNSS-M	180,200		leaves	868
Glc	2,3,4,6; 2,3,4; 2,3,6; 2,4,6; 3,4,6; 3; 6	3% NPGS	160-230	2	model	394a
Gal	4					
Rha	2,3,4					
Ara	2,3,5	5% LAC 4R-886	187		<i>Heteropogon contortus</i> (spear grass)	429
Xyl	2,3,4; 2,3; 2; 3				<i>Cynodon plectostachyus</i> (giant star grass)	753a

TABLE XXIX
Periodate Oxidation of Methylated Sugars

<i>Methyl ethers of</i>	<i>Column of</i>	<i>Column temp. (°C)</i>	<i>Rate (deg. min⁻¹)</i>	<i>Material or problem studied</i>	<i>References</i>
Arabinose	13% Versamid			synthetic arabinan	875
Glucose				<i>Mycobacterium phlei</i>	789
				gum ghatti	871,1091
	5% SE-30	145		"polymaltose"	642
				<i>Khaya ivorensis</i>	1092
Galactose	10% Versamid	110-220	8	synthetic galactan	207
				<i>Acacia nubica</i>	637
				centrosema seed	869
				<i>Mycobacteria</i>	870
				<i>Acacia</i> gums	106
				gum ghatti	1091
Gulose	10% poly(phenyl ether)	200			
	3% XE-60	150		alginic acid	88
Mannose	12% Versamid	160	3 min	synthetic mannan	640
			6,8,10		
	10% poly(phenyl ether)	200		gum ghatti	1091
				synthesis	873
	10% Carbowax 6000			dermatophytes	872
	15% BDS	175		gum ghatti	871
	3% XE-60	150		alginic acid	88,595
Rhamnose	10% Versamid	80-200	8	synthetic rhamnan	641
		raised to 230	after 16 min		
Heptose		140-240	10	lipopolysaccharide	874

TABLE XXX
Miscellaneous Ethers

<i>Methyl ethers of</i>	<i>Column of</i>	<i>Column temp. (°C)</i>	<i>Rate (deg. min⁻¹)</i>	<i>Material or problem studied</i>	<i>References</i>
<i>O</i> -(2-Hydroxyethyl)glucoses	3% QF-1	210,250		synthesis cellulose	901 1151,1152
	2% SE-52	150-250	3	starch	876
	5% BDS	90-210	2	starch and dextran	1152a
<i>2-O</i> -(2-Hydroxyethyl)glucoses	5% SE-30	190		synthesis inner glycosides	877 1152b
<i>O</i> -Allylglucoses	19% SE-52	200		synthesis and cellulose	1153
		180-220	0.5		1154
	8.5% XE-60	125 80-140	0.5		
<i>O</i> -(Methylsulfonyl)ethylglucoses	8.5% XE-60	200		synthesis	1155
		125-225	10		
	3% SE-52 (10% Apiezon M; 3% QF-1)	125-250	10	cellulose	1154,1156,1157
<i>O</i> -(2-Aminoethyl)glucoses	5% SE-30	185,190		synthesis and cellulose cellulose	877 1158
<i>O</i> -(2-Diethylaminoethyl)glucoses	8.5% XE-60	140		synthesis and cellulose	1159,1160
	19% SE-52	210			
	(QF-1, Apiezon M, SE-30, Carbowax 20 M)			cellulose and starch	1161
<i>O</i> -[<i>N</i> -Methyl- <i>N</i> -(2-hydroxyethyl)-amino]ethylglucoses	1% SE-30	185		synthesis	1162
	2% OV-3				

TABLE XXXI
Gas-liquid Chromatography-Mass Spectrometry in Structural Investigations

<i>Compounds</i>	<i>References</i>	<i>Compounds</i>	<i>References</i>
<i>A. Trimethylsilyl compounds</i>		<i>B. Alditol acetates</i>	
Methyl glycosides	210,701 773 818	Model studies	919,920,921
Acids and lactones	261,499,500,502,504,508, 522,535,845	General	734,863
Amino sugars	144,200,903,915	Monosaccharides	
Hydroxyethyl ethers	901	3- <i>O</i> -Methylxylose	711
Disaccharides	389,909,911a,912,912a, 913,914,915,1163	Dimethylmannoses	825
Smith-degraded	787	<i>O</i> -Methylabequose	833
Trisaccharides	910,911,927	Disaccharides	767,844,1127,1132,1134, 1135
		Oligosaccharides	712,713,720
		Arabinans	1031a
		Xylans	
		red cotton-wood	685

(Continued)

TABLE XXXI (Continued)

Compounds	References	Compounds	References
<i>B. (Continued)</i>		Lipopolysaccharides	435,437-440,727,730,832, 1045
<i>Eucalyptus globulus</i>	1032	O antigens	
<i>Betula verrucosa</i>	1032	(D) ^a	712
<i>Pinus radiata</i>	762,1149	(CD ₂ ,D) ^a	713
Glucans		(CD ₃) ^a	432,787,1044
fungal	763	M antigens	443
hetero (D) ^a	922		444
(D) ^a	781b		883
bacterial (CD ₃) ^a	445		
dextran	749	<i>C. Permethylated Alditols</i>	
dextran (Eth) ^a	765	Disaccharides	911a,925,926
algae	865,866	Trisaccharides	928
Glucomannans	762		
<i>Picea mariana</i> and	793	<i>D. Aldononitriles</i>	
<i>Picea sitchensis</i>			394a
Capsular			
<i>Klebsiella</i>	720,861,862,1047-1049, 1052-1054		
<i>Pneumococcus</i> (D) ^a	484a		

^a D = deuterated samples; CD₂ = methylation with CD₃I; Eth = methyl and ethyl ethers.

TABLE XXXII
Recommended Weights of Packing*

Mesh size of support	Type of metal tubing	O.D. (inches) of tubing	Supports used (grams of packing/ft of column length)				
			GAS CHROM S.A.P.Z.Q	GAS CHROM R.R.A.R.P.R.Z and Chromo- sorb P	Chromosorb W	Chromosorb G	Porous polymer beads ^b
45-60	SS	1/8	0.45 ^c	0.55	0.5	—	—
		3/16	—	2.1	—	—	—
		1/4	—	4.0 ^d	2.8 ^c	4.6	—
60-80	SS	1/8	0.4	0.6	0.4	0.75	0.5
		1/4	2.4	3.5	2.8	4.6	2.5
80-100	SS	1/8	0.45	0.6	0.45	0.8	0.5
		1/4	2.7	3.7	2.9	4.7	2.6
100-120	SS	1/8	0.5	0.6	0.5	0.8	0.5
		1/4	2.7	3.7	2.9	4.7	2.8 ^c

(Continued)

TABLE XXXII (Continued)

Mesh size of support	Type of metal tubing	O.D. (inches) of tubing	Supports used (grams of packing/ft of column length)				
			GAS CHROM S.A.P.Z.Q	GAS CHROM R.R.A.R.P.RZ and Chromo- sorb P	Chromosorb W	Chromosorb G	Porous polymer beads ^b
45-60	Al	1/8	—	0.4 ^d	0.3 ^d	—	—
60-80	Al	1/8	0.25 ^c	0.4	0.25 ^c	—	—
		1/4	1.4	2.8 ^c	—	3.2	—
		3/8	5.1	6.8 ^d	—	10.3	—
80-100	Al	1/8	0.3	0.4	0.3 ^c	—	—
		1/4	1.7	—	—	—	—
		3/8	5.3 ^c	—	—	10.3	—
100-120	Al	1/8	0.3	0.45 ^c	—	—	—
		1/4	1.7 ^c	—	—	—	1.6 ^d
		3/8	5.3 ^c	—	—	—	5.0
45-60	Cu	1/4	—	3.4	—	—	—
60-80	Cu	1/8	0.3	0.35 ^d	—	—	—
		1/4	1.6	3.2 ^d	—	—	—
80-100	Cu	1/8	0.3	0.45 ^c	0.3	—	—

^a Reproduced by kind permission of Applied Science Laboratories, Inc.^b Porous polymer beads must be packed tightly; figures are thought to be minimum acceptable value.^c Estimated.^d Limited data.

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- (1143) A. M. Stephen, *Carbohydr. Res.*, **5**, 335 (1967).
- (1144) N. Roy and T. E. Timell, *Carbohydr. Res.*, **6**, 482 (1968).
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- (1157) S. P. Rowland, V. O. Cirino, and A. L. Bullock, *Can. J. Chem.*, **44**, 1051 (1966).
- (1158) E. J. Roberts and S. P. Rowland, *Text. Res. J.*, **39**, 686 (1969).
- (1159) E. J. Roberts and S. P. Rowland, *Can. J. Chem.*, **45**, 261 (1967).
- (1160) S. P. Rowland, E. J. Roberts, and C. P. Wade, *Text. Res. J.*, **39**, 520 (1969).
- (1161) E. J. Roberts and S. P. Rowland, *Carbohydr. Res.*, **5**, 1 (1967).
- (1162) E. J. Roberts and S. P. Rowland, *Can. J. Chem.*, **48**, 1383 (1970).
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STRUCTURES AND SYNTHESSES OF AMINOGLYCOSIDE ANTIBIOTICS

BY SUMIO UMEZAWA

*Department of Applied Chemistry, Faculty of Engineering, Keio University,
Hiyoshi, Yokohama, Japan*

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I. INTRODUCTION

A great number of antibiotics composed essentially of carbohydrates have been obtained from micro-organisms and called aminoglycoside antibiotics because they generally contain several amino groups in their glycosidic moieties. The remarkably successful clinical application of this group of antibiotics aroused widespread interest and stimulated intensive investigation of them by organic chemists, leading to marked advances in several areas of carbohydrate chemistry. Streptomycin, discovered by Waksman and coworkers in 1944, was the first aminoglycoside antibiotic. Remarkable developments have since been made in this field of antibiotics. Many new aminoglycoside antibiotics have been discovered and their structures elucidated, as shown in Table I.

TABLE I
The Dates of Discovery of Aminoglycoside Antibiotics

<i>Year</i>	<i>Antibiotic</i>	<i>Year</i>	<i>Antibiotic</i>
1944	Streptomycin	1967	α -D-Mannosyl 2-amino-2-deoxy- α -D-glucoside
1947	Mannosidostreptomycin	1969	N-Demethylstreptomycin
1949	Hydroxystreptomycin		Hybrimycins
	Neomycins	1970	Ribostamycin
1956	Trehalosamine		Sisomicin
1957	Kanamycins		A-396-I
1958	Hygromycin B	1971	Butirosins
1959	Paromomycins		Lividomycins
1961	Spectinomycin		Tobramycin
1963	Bluensomycin		Validamycins
	Gentamicins	1973	Apramycin
1965	Kasugamycin		Bu-1709 E ₁ , E ₂
	Destomycin A		SS-56C

The synthetic challenge posed by the complex carbohydrate antibiotics was met by the total synthesis of the kanamycins in 1968, and, later, ribostamycin, butirosin B, and tobramycin were synthesized. The total synthesis of dihydrostreptomycin was accomplished by Umezawa and coworkers in 1973.

Hand in hand with these studies, the mechanisms of inactivation of aminoglycoside antibiotics by resistant bacteria have been revealed by medical microbiologists in Japan and the U. S. A. The combination of the synthetic developments and the mechanistic view of drug resistance has opened the way to the synthesis of new and improved antibiotics that are remarkably effective against micro-organisms resistant to the natural aminoglycoside antibiotics.

Excellent reviews of aminoglycoside antibiotics¹⁻³ have appeared. The chemistry of individual sugars present in this kind of antibiotic has been reviewed,⁴⁻⁷ along with that of sugars contained in other kinds of antibiotics. Because the last article on aminoglycoside antibiotics that ap-

- (1) J. D. Dutcher, *Advan. Carbohydr. Chem.*, **18**, 259 (1963).
- (2) H. Umezawa, "Recent Advances in Chemistry and Biochemistry of Antibiotics," Microbial Chemistry Research Foundation, Tokyo, 1964, p. 67.
- (3) S. Hanessian and T. H. Haskell, in "The Carbohydrates; Chemistry and Biochemistry," W. Pigman and D. Horton, eds., Academic Press, Inc., New York, N. Y., 2nd Edition, 1970, Vol. IIA, p. 159.
- (4) A. B. Foster and D. Horton, *Advan. Carbohydr. Chem.*, **14**, 213 (1959).
- (5) L. Hough and A. C. Richardson, in "Rodd's Chemistry of Carbon Compounds," S. Coffey, ed., Elsevier Publishing Co., Amsterdam, The Netherlands, 2nd Edition, 1967, Vol. 1F, p. 448.

peared in this Series, that by Dutcher,¹ was published in 1963, the present Chapter will cover, in outline, the key literature since that time, placing major emphasis on recent advances.

The classification of aminoglycoside antibiotics in this Chapter will be based on overall, common structural relationships, being divided into the seven groups discussed in Section II.

It should be appreciated that modern instrumental techniques, particularly nuclear magnetic resonance (n.m.r.) spectroscopy (including ¹³C n.m.r. spectroscopy), X-ray crystal-structure analysis, infrared (i.r.) spectroscopy, mass spectrometry (m.s.), optical rotatory dispersion (o.r.d.), circular dichroism (c.d.), and Reeves's copper-complexing method have greatly contributed to the advances already mentioned. Studies dealing extensively with the mass spectrometry,⁸⁻¹⁰ proton magnetic resonance (p.m.r.) spectroscopy,¹¹ Reeves's copper-complexing method,¹² a modification of the copper-complexing method,¹³ gas-liquid chromatography (g.l.c.),¹⁴⁻¹⁶ thin-layer chromatography (t.l.c.),¹⁷ and paper electrophoresis¹⁸ of aminoglycosides have also been reported.

The mechanisms of inactivation of aminoglycoside antibiotics by resistant organisms, and the chemistry of the inactivated products, are described in Chapter 4 of this Volume (see p. 183).

II. STRUCTURES OF AMINOGLYCOSIDE ANTIBIOTICS

1. Streptomycins and Bluensomycin

In the streptomycin group, a unique branched-chain sugar, streptose or its reduced or oxidized form, is present, and the aglycon is streptidine,

-
- (6) D. Horton, in "The Amino Sugars," R. W. Jeanloz, ed., Academic Press Inc., New York, N. Y., 1969, Vol. 1A, p. 3.
 - (7) J. S. Brimacombe, *Angew. Chem., Int. Ed. Engl.*, **10**, 236 (1971).
 - (8) P. J. L. Daniels, M. Kugelman, A. K. Mallams, R. W. Tkach, H. F. Vernay, J. Weinstein, and A. Yehaskel, *Chem. Commun.*, 1629 (1971).
 - (9) S. Hanessian, *Methods Biochem. Anal.*, **19**, 105 (1971).
 - (10) S. Inouye, *Chem. Pharm. Bull. (Tokyo)*, **20**, 2331 (1972).
 - (11) H. Naganawa, S. Kondo, K. Maeda, and H. Umezawa, *J. Antibiot. (Tokyo)*, **24**, 823 (1971).
 - (12) C. B. Barlow and R. D. Guthrie, *J. Chem. Soc. (C)*, 1194 (1967).
 - (13) S. Umezawa, T. Tsuchiya, and K. Tatsuta, *Bull. Chem. Soc. Jap.*, **39**, 1235 (1966).
 - (14) K. Tsuji and J. H. Robertson, *Anal. Chem.*, **41**, 1332 (1969).
 - (15) K. Tsuji and J. H. Robertson, *Anal. Chem.*, **42**, 1661 (1970).
 - (16) S. Omoto, S. Inouye, and T. Niida, *J. Antibiot. (Tokyo)*, **24**, 430 (1971).
 - (17) J. K. Pauncz, *J. Antibiot. (Tokyo)*, **25**, 677 (1972).
 - (18) K. Maeda, A. Yagi, H. Naganawa, S. Kondo, and H. Umezawa, *J. Antibiot. (Tokyo)*, **22**, 635 (1969).

except for bluensomycin in which the aglycon is bluensidine (glebidine). The latter antibiotic was reported by two research groups under the names of glebomycin¹⁹ and bluensomycin.²⁰

Whereas streptidine and 2-deoxy-2-(methylamino)-L-glucose, components of streptomycin, were synthesized by 1950, some fifteen years elapsed before the synthesis of streptose was achieved by Dyer and coworkers.²¹ Another synthesis of streptose was reported by Paulsen and coworkers.²² The glycosidic linkage between streptose and 2-deoxy-2-(methylamino)-L-glucose had previously been shown to be α -L, and the glycosidic linkage between streptose and streptidine has been revised to be α -L on the basis of n.m.r.-spectral studies.^{23,24}

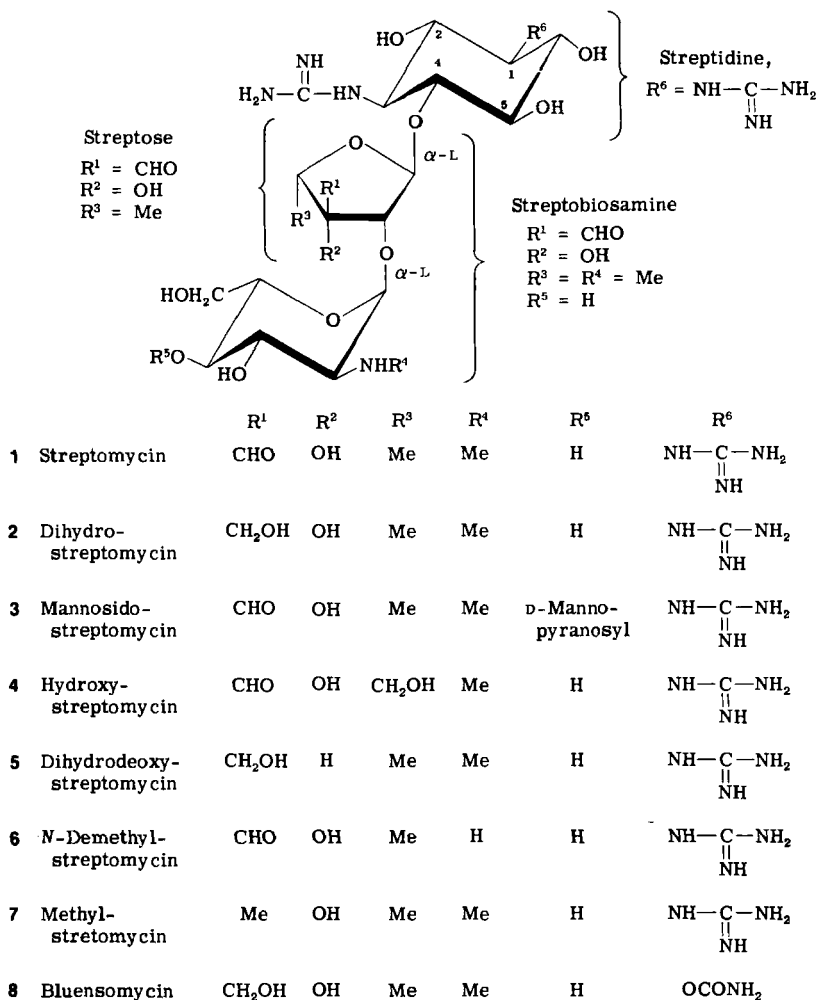
The absolute configuration of the streptidine moiety was determined by Dyer and A. W. Todd²⁵ by applying Reeves's copper-complexing method²⁶ to *N,N'*-diacetyl-4-deoxystreptamine, derived from streptomycin by degradation. Moreover, the same conclusion was reached by Tatsuoka and coworkers^{27,28} on applying the Reeves method to 2,6-di-*O*-methylstreptamine derived from dihydrostreptomycin; this revealed that the streptobiosamine moiety is attached to C-4 in the *R* configuration. The absolute structure of streptomycin was confirmed by an X-ray analysis of streptomycin oxime selenate.²⁹

The conformational structures for streptomycin and related antibiotics can now be written as shown by formulas 1 to 8.

Bluensomycin had been shown to be an analog of dihydrostreptomycin in which the streptidine moiety of the latter is replaced by bluensidine; however, its absolute configuration was not established. Barlow and Anderson³⁰ made a polarimetric study of the interaction of the tetra-aminecopper reagent of Umezawa and coworkers¹³ with bluensomycin,

- (19) T. Miyaki, H. Tsukiura, M. Wakae, and H. Kawaguchi, *J. Antibiot.* (Tokyo), **A15**, 15 (1962).
- (20) B. Bannister and A. D. Argoudelis, *J. Amer. Chem. Soc.*, **85**, 234 (1963).
- (21) J. R. Dyer, W. E. McGonigal, and K. C. Rice, *J. Amer. Chem. Soc.*, **87**, 654 (1965).
- (22) H. Paulsen, V. Sinnwell, and P. Stadler, *Angew. Chem., Int. Ed. Engl.*, **11**, 149 (1972).
- (23) I. J. McGilveray and K. L. Rinehart, Jr., *J. Amer. Chem. Soc.*, **87**, 4002 (1965).
- (24) P. Claes, H. Vanderhaeghe, J. Totte, and G. Slinchx, *Bull. Soc. Chim. Belges*, **78**, 151 (1969).
- (25) J. R. Dyer and A. W. Todd, *J. Amer. Chem. Soc.*, **85**, 3896 (1963).
- (26) R. E. Reeves, *Advan. Carbohydr. Chem.*, **6**, 107 (1951).
- (27) S. Tatsuoka and S. Horii, *Proc. Jap. Acad.*, **39**, 314 (1963).
- (28) S. Tatsuoka, S. Horii, K. L. Rinehart, Jr., and T. Nakabayashi, *J. Antibiot.* (Tokyo), **A17**, 88 (1964).
- (29) S. Neidle, D. Rogers, and M. B. Hursthouse, *Tetrahedron Lett.*, 4725 (1968).
- (30) C. B. Barlow and L. Anderson, *J. Antibiot.* (Tokyo), **25**, 281 (1972).

its deamidinodecarbamoyl derivative, and several reference compounds of the streptomycin series, and the results supported the absolute structure depicted in formula 8.



Two forms (α and β) of streptomycin salts are known. β -Salts are crystalline, sparingly soluble in water, and different from ordinary amorphous salts (α -salts). In the β -form, the aldehyde group was thought to be bound by cyclization with the methylamino group.^{31,32} In

(31) L. J. Heusen, M. A. Dolliven, and E. T. Stiller, *J. Amer. Chem. Soc.*, **75**, 4013 (1953).

(32) D. P. Young, *J. Chem. Soc.*, 1337 (1961).

fact, n.m.r.-spectral studies of streptomycin and dihydrostreptomycin in deuterium oxide suggested³³ that streptomycin forms an aldehyde ammonia derivative ($-\text{NCH}_3-\text{CHOH}-$) which is in a second equilibrium with a quaternary iminium form ($-\overset{\oplus}{\text{NCH}_3}=\text{CH}-$).

The fermentation of streptomycin was modified by Heding³⁴ to yield *N*-demethylstreptomycin (6). Mannosidohydroxystreptomycin was obtained by fermenting with a *Nocardia* strain.³⁵

The chemically modified products 5 and 7 will be mentioned in Section IV,2 (see p. 178).

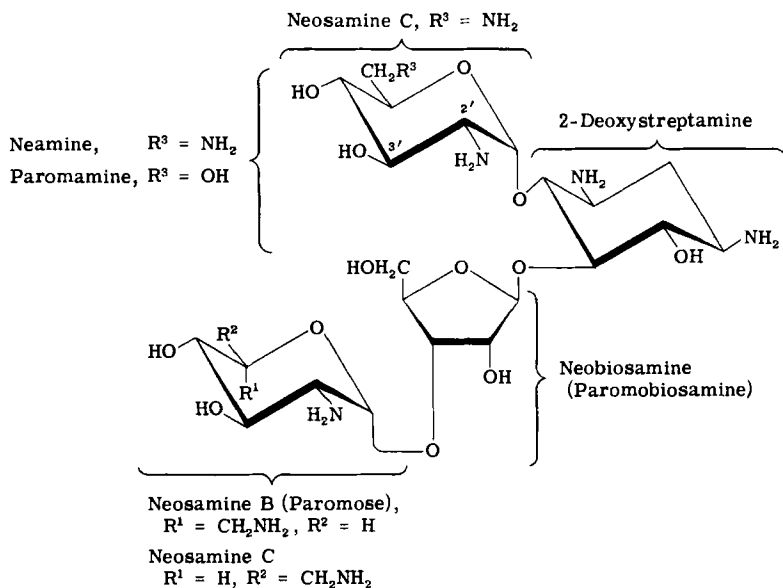
2. Neomycins, Paromomycins, Hybrimycins, and Lividomycins

a. Neomycins and Paromomycins.—Neomycin A is identical to neamine, which is a common degradation product of neomycins B (9) and C (10). Neomycin B and neomycin C differ only in the stereochemistry of the aminomethyl group attached to C-5 in a diaminodideoxy sugar residue. Neomycins LP_B and LP_C are monoacetyl derivatives of neomycins B and C at the C-3 amino group of the 2-deoxystreptamine moiety.³⁶ The component sugars, neosamine B (Ref. 37) (paromose, 2,6-diamino-2,6-dideoxy-L-idose), neosamine C (Refs. 37–40) (2,6-diamino-2,6-dideoxy-D-glucose), and 2-deoxystreptamine^{41–43} had been synthesized by 1965.

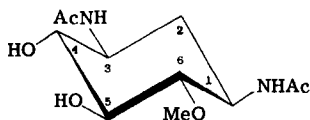
The attachment of the neosamine group to C-3 of the D-ribose residue and the linkage of the neobiosamine group to C-5 of 2-deoxystreptamine

- (33) J. Aronson, W. L. Meyer, and T. D. Brock, *Nature*, **202**, 555 (1964).
- (34) H. Heding, *Acta Chem. Scand.*, **23**, 1275 (1969).
- (35) Societa Farmaceutici Italia, Brit. Pat. 955,762 (Apr. 22, 1964); *Chem. Abstr.*, **61**, 2440 (1964).
- (36) K. L. Rinehart, Jr., "The Neomycins and Related Antibiotics," E. R. Squibb Lectures on Chemistry of Microbial Products, John Wiley & Sons, Inc., New York, N. Y., 1964, p. 93.
- (37) W. Meyer zu Reckendorf, *Chem. Ber.*, **96**, 2017 (1963).
- (38) H. Weidemann and H. K. Zimmerman, Jr., *Angew. Chem.*, **72**, 750 (1960).
- (39) H. Weidemann and H. K. Zimmerman, Jr., *Ann.*, **684**, 226 (1965).
- (40) K. L. Rinehart, Jr., M. Hichens, K. Streigler, K. R. Rover, T. P. Culbertson, S. Tatsuoka, S. Horii, T. Yamaguchi, H. Hitomi, and A. Miyake, *J. Amer. Chem. Soc.*, **83**, 2964 (1961).
- (41) M. Nakajima, A. Hasegawa, and N. Kurihara, *Tetrahedron Lett.*, 967 (1964); *Ann.*, **689**, 235 (1965).
- (42) T. Suami, S. Ogawa, and H. Sano, *Tetrahedron Lett.*, 2671 (1967); *Bull. Chem. Soc. Jap.*, **41**, 1014 (1968).
- (43) D. Dijkstra, *Rec. Trav. Chim.*, **87**, 161 (1968).

were established by methylation and hydrolysis studies,⁴⁴ and evidence for the configurations of the anomeric linkages were obtained from n.m.r. studies.⁴⁵ Furthermore, Hichens and Rinehart⁴⁶ first introduced Reeves's copper-complexing method²⁶ to determine the absolute stereochemistry of the neomycins and the paromomycins (11 and 12). 1,3-Di-*N*-acetyl-6-*O*-methyl-2-deoxystreptamine (13), derived from neomycin B



	R^1	R^2	R^3
9 Neomycin B	CH_2NH_2	H	NH_2
10 Neomycin C	H	CH_2NH_2	NH_2
11 Paromomycin I	CH_2NH_2	H	OH
12 Paromomycin II	H	CH_2NH_2	OH



1,3-Di-*N*-acetyl-6-*O*-methyl-2-deoxystreptamine

13

- (44) K. L. Rinehart, Jr., M. Hichens, A. D. Argoudelis, W. S. Chilton, H. E. Carter, M. P. Georgiadis, C. P. Schaffner, and R. T. Schillings, *J. Amer. Chem. Soc.*, **84**, 3218 (1962).

and paromomycin I, had a high positive increment, indicating the absolute configuration of the unsymmetrically substituted 2-deoxystreptamine portion of these antibiotics. [The numbering of the positions in the substituted 2-deoxystreptamine follows the proposal by Hichens and Rinehart,⁴⁶ and this numbering has been rationalized⁴⁸ (see also, Ref. 66, p. 123)].

In the paromomycins, paromobiosamines I and II are identical with neobiosamines B and C, respectively, and paromose in paromomycin I is identical with neosamine B. The configurations of the glycosidic linkages and the absolute stereochemistry of the 2-deoxystreptamine moiety were determined by n.m.r.-spectral studies⁴⁵ and by application⁴⁸ of Reeves's copper-complexing method.²⁸ Mass-spectral studies^{9,47} also supported the sequential arrangement and gross structures of the units of which the paromomycins are composed.

Paromamine was isolated⁴⁸ from a crude kanamycin complex. D-Mannosylparomomycin, in which a D-mannopyranosyl group is attached to O-4 of the paromose (neosamine B) by an α -D linkage, was isolated from a culture of a *Streptomyces* strain that produces lividomycins and paromomycin.⁴⁹

Neamine and paromamine were synthesized by Umezawa and co-workers (see pp. 140 and 142).

b. Hybrimycins.—Rinehart and coworkers⁵⁰ reported the discovery of the hybrimycins, which are closely related to the neomycins. These antibiotics (14–17) contain streptamine or a stereoisomer thereof instead of 2-deoxystreptamine. As a study by Matsukawa and Tanaka⁵¹ on the mechanism of action suggested that the 2-deoxystreptamine moiety of neomycins and paromomycins is responsible for the codon-misreading activity of these antibiotics, variation of the 2-deoxystreptamine ring was undertaken. A mutant of *Streptomyces fradiae* incapable of synthesizing neo-

(45) K. L. Rinehart, Jr., W. S. Chilton, M. Hichens, and M. von Phillipsborn, *J. Amer. Chem. Soc.*, **84**, 3216 (1962).

(46) M. Hichens and K. L. Rinehart, Jr., *J. Amer. Chem. Soc.*, **85**, 1547 (1963).

(47) D. C. DeJongh, J. D. Hribar, S. Hannessian, and P. W. K. Woo, *J. Amer. Chem. Soc.*, **89**, 3364 (1967).

(48) M. Murase, T. Ito, S. Fukatsu, and H. Umezawa, in "Progress in Antimicrobial and Cancer Chemotherapy," Proc. 6th Int. Congr. Chemother., Univ. of Tokyo Press, 1970, Vol. II, p. 1098.

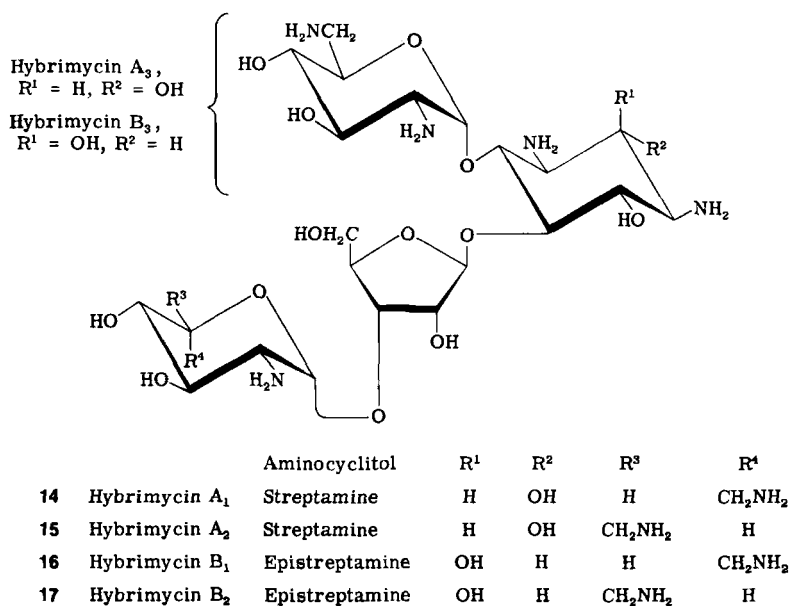
(49) T. Mori, Y. Kyotani, I. Watanabe, and T. Oda, *J. Antibiot.* (Tokyo), **25**, 317 (1972).

(50) W. T. Shier, K. L. Rinehart, Jr., and D. Gottlieb, *Proc. Nat. Acad. Sci. U. S.*, **63**, 198 (1969).

(51) H. Matsukawa and N. Tanaka, *J. Antibiot.* (Tokyo), **21**, 70 (1968).

mycin in the absence of added 2-deoxystreptamine was isolated, and, by utilizing the mutant, hybrimycin A and B were obtained by feeding streptamine and 2-epistreptamine (a portion of spectinomycin), respectively, to growing cultures of the mutant. The structures of the hybrimycins were established by mass-spectral, n.m.r., and optical rotatory studies on their appropriate derivatives. The antibiotic activity of hybrimycin A is approximately the same as that of neomycin, and it is more active than hybrimycin B.

Selective hydrolysis of hybrimycin A and B gave hybrimycin A₃ and B₃, respectively, which are less active than the closely related neamine⁵² (83, see p. 142).

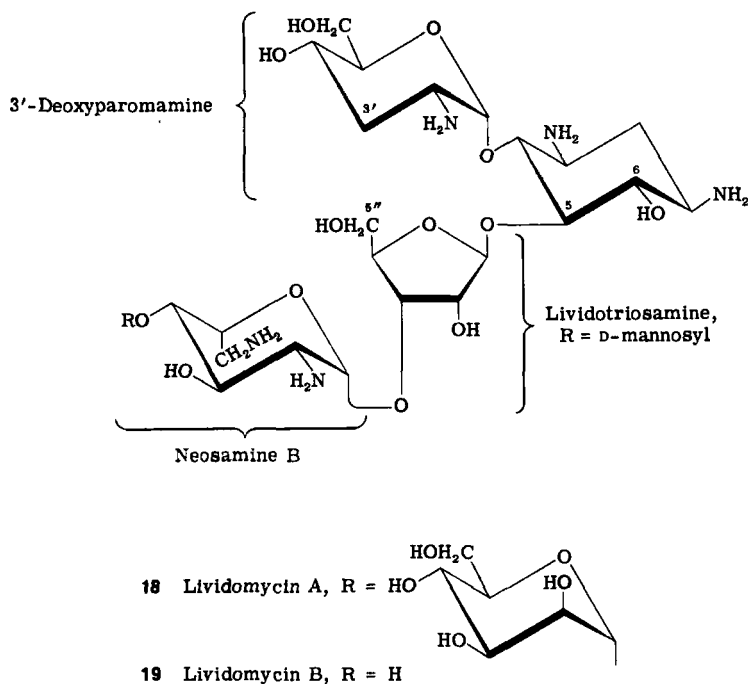


c. **Lividomycins.**—Mori and colleagues⁵³ reported the discovery of the lividomycins, which are related to the paromomycins. An antibiotic complex is produced by *Streptomyces lividus*; it consists of lividomycins A (18) and B (19), D-mannosidoparomomycin, and paromomycin I. All of the components are active against Gram-positive and Gram-negative bac-

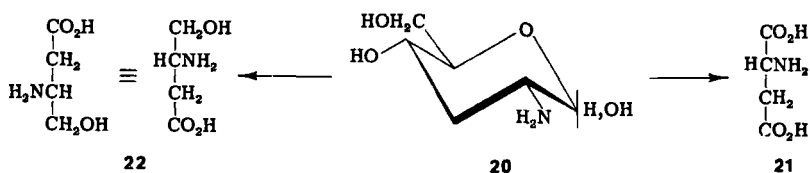
(52) W. T. Shier, K. L. Rinehart, Jr., and D. Gottlieb, *J. Antibiot.* (Tokyo), **23**, 51 (1970).

(53) T. Mori, T. Ichianagi, H. Kondo, K. Tokunaga, and T. Oda, *J. Antibiot.* (Tokyo), **24**, 339 (1971).

teria, including *Mycobacteria*. Lividomycin A (18) is D-mannosyllividomycin B.



Methanolysis of lividomycin A gave a C_{12} amine and methyl lividotriosaminide.⁵⁴ Acetylation of the former product gave a tri-*N*-acetyl derivative which, on hydrolysis, yielded 2-deoxystreptamine and an aminohexose. By successive periodate oxidation, bromine-water oxidation, and acid hydrolysis, the *N*-acetyl derivative of the aminohexose gave D-aspartic acid (21). The aminohexose also led to a 3-amino-4-hydroxybutyric acid (22). N.m.r.-spectral studies of the peracetylated methyl glycoside of the aminohexose indicated that it is 2-amino-2,3-dideoxy-D-ribo-hexose (20), and this structure was confirmed by com-



(54) T. Oda, T. Mori, and Y. Kyotani, *J. Antibiot.* (Tokyo), **24**, 503 (1971).

parison with a synthetic specimen.⁵⁵ The α -D glycosidic linkage between **20** and 2-deoxystreptamine was shown by the n.m.r. spectrum of the C₁₂-amine, which is, therefore, 3'-deoxyparomamine.

Methylation and hydrolysis studies of penta-*N*-acetyl-lividomycin A resulted in the isolation of 1-*O*-acetyl-2,3,4,6-tetra-*O*-methyl- α -D-mannose and 1,3-di-*O*-acetyl-2,5-di-*O*-methyl- β -D-ribose, indicating that the D-mannosyl group is terminal in lividotriosamine. Controlled hydrolysis of methyl *N,N'*-diacetyl-lividotriosaminide, followed by acetylation, yielded D-mannosyl-*N,N'*-diacetylneosome B. Lividotriosaminol, obtained from lividotriosamine through reduction with borohydride yielded, on hydrolysis, D-mannose and neosome B, but no D-ribose. These results showed that, in lividotriosamine, C-1 of neosome B is linked to O-3 of D-ribose, and O-1 of the D-ribose residue is unsubstituted.⁵⁶ Methylation and hydrolysis of penta-*N*-acetyl-lividomycin A also gave 1,3-di-*N*-acetyl-6-*O*-methyl-2-deoxystreptamine (**13**), indicating that lividotriosamine is attached to O-5 of 2-deoxystreptamine. Evidence for the configurations of the anomeric linkages in lividomycin A was obtained from n.m.r.-spectral studies (by reference to those on neomycins⁴⁵) and from optical rotatory considerations.

The structure of lividomycin B was established⁵⁷ by conversion of lividomycin A into lividomycin B by means of the Barry degradation.

3. Kanamycins and Tobramycin

a. **Kanamycins.**—The structures of kanamycins A (**23**), B (**24**), and C (**25**) had been established by 1964, except for the absolute sequence of substitution on the 2-deoxystreptamine moiety.^{2,58,59} Their structural relationships are shown by formulas **23** to **25**. Acid hydrolysis of kanamycin A gave 2-deoxystreptamine and two amino sugars, namely, 3-amino-3-deoxy-D-glucose (kanosamine) and 6-amino-6-deoxy-D-glucose; this was their first isolation from a natural source. The glycosidic linkage between kanosamine and 2-deoxystreptamine in kanamycin A is more resistant to acid hydrolysis than that between the latter and 6-amino-6-deoxy-D-glucose, because of the proximity of the amino group in kanosamine to the glycosidic linkage. Controlled hydrolysis of kanamycin A gave two

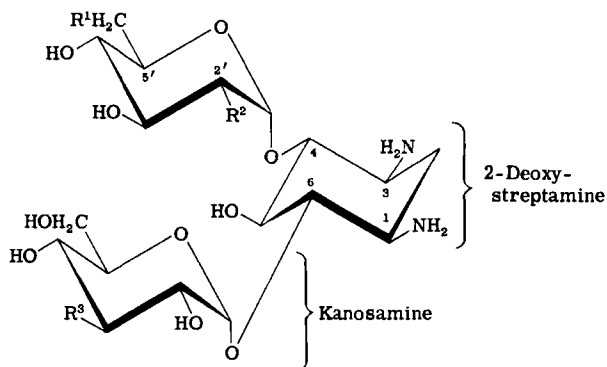
(55) W. Meyer zu Reckendorf and W. A. Bonner, *Tetrahedron*, **19**, 1711 (1963).

(56) T. Oda, T. Mori, and Y. Kyotani, *J. Antibiot.* (Tokyo), **24**, 511 (1971).

(57) T. Mori, Y. Kyotani, I. Watanabe, and T. Oda, *J. Antibiot.* (Tokyo), **25**, 149 (1972).

(58) H. Umezawa, *Asian Med. J.*, **11**, 69 (1968).

(59) K. Maeda, "Streptomyces Products Inhibiting Mycobacteria," E. R. Squibb Lectures on Chemistry of Microbial Products, John Wiley & Sons, Inc., New York, N. Y., 1965, p. 51.

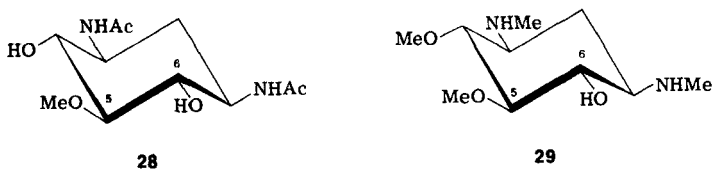


- 23** Kanamycin A, $R^1 = \text{NH}_2$, $R^2 = \text{OH}$, $R^3 = \text{NH}_2$
24 Kanamycin B, $R^1 = R^2 = R^3 = \text{NH}_2$
25 Kanamycin C, $R^1 = \text{OH}$, $R^2 = R^3 = \text{NH}_2$
26 NK-1001, $R^1 = \text{NH}_2$, $R^2 = R^3 = \text{OH}$
27 NK-1012-1, $R^1 = R^2 = \text{NH}_2$, $R^3 = \text{OH}$

pseudodisaccharides composed of 2-deoxystreptamine and one of the amino sugars.^{60,61} Hydrolysis of permethylated, *N*-acetylated kanamycin A gave 1,3-di-*N*-acetyl-5-*O*-methyl-2-deoxystreptamine (28) which was optically inactive.⁶² Periodate oxidation of kanamycin A, followed by hydrolysis, yielded 2-deoxystreptamine only.⁶³ These results indicated the C-4,6 substitution pattern in the 2-deoxystreptamine. The absolute sequence of the C-4,6 substitution was first deduced by Hichens and Rinehart⁴⁶ from their studies on neomycin using Reeves's copper-complexing method;²⁶ the presence of paromamine in kanamycin C suggested the presence of another amino sugar group, attached at O-6 of 2-deoxystreptamine. Tatsuoka and coworkers²⁸ confirmed the absolute sequence by application of the Reeves method to the tri-*N*-acetyl derivative of (3-amino-3-deoxy- α -D-glucosyl)-2-deoxystreptamine⁶⁰ obtained from kanamycin A by partial hydrolysis. On the other hand, Umezawa and coworkers⁶⁴ applied their tetraamminecopper method¹³ to 1,3-di-*N*-

- (60) K. Maeda, M. Murase, H. Mawatari, and H. Umezawa, *J. Antibiot.* (Tokyo), **A11**, 163 (1958).
 (61) S. Umezawa and T. Tsuchiya, *J. Antibiot.* (Tokyo), **A15**, 73 (1962).
 (62) S. Umezawa, Y. Ito, and S. Fukatsu, *J. Antibiot.* (Tokyo), **A11**, 120, 160 (1958).
 (63) M. J. Cron, O. B. Fardig, D. L. Johnson, D. F. Whitehead, I. R. Hooper, and R. U. Lemieux, *J. Amer. Chem. Soc.*, **80**, 4115 (1958).
 (64) S. Umezawa, K. Tatsuta, and T. Tsuchiya, *Bull. Chem. Soc. Jap.*, **39**, 1244 (1966).

methyl-4,5-di-*O*-methyl-2-deoxystreptamine (29), derived from the (3-amino-3-deoxy- α -D-glucosyl)-2-deoxystreptamine, and reached to the same conclusion. The two α -D anomeric linkages were indicated by optical rotatory and i.r.-spectral data.⁶⁵



The 100-MHz n.m.r. spectrum of kanamycin A in deuterium oxide was discussed by Lemieux and coworkers;⁶⁶ the chemical shift at higher field for one of the two anomeric protons was assigned to the anomeric proton of the 6-*O*-glycosyl group, and that at lower field, to that of the 4-*O*-glycosyl group. In addition, they discussed the conformation of kanamycin A in aqueous solution. Nakajima and coworkers⁶⁷ examined the n.m.r. spectra of *N*-acetyl derivatives of kanamycin and related compounds, and concluded that the signal of the anomeric proton of the glycosyl group at O-4 of 2-deoxystreptamine generally shifts to lower field by anisotropy of the *N*-acetyl carbonyl group of 1,3-di-*N*-acetyl-2-deoxystreptamine or *N,N'*-diacetylstreptamine.

Kanamycin B (24) and C (25) differ in that the 6-amino-6-deoxy-D-glucosyl group in kanamycin A is replaced by a 2,6-diamino-2,6-dideoxy-D-glucosyl group and by a 2-amino-2-deoxy-D-glucosyl group, respectively.^{68,69} The former amino sugar is also contained in the neomycins, and the latter in the paromomycins. Furthermore, other kanamycins, namely, NK-1001 (26) and NK-1012-1 (27), were isolated⁴⁸ from the cultures of mutants of *Streptomyces kanamyceticus*.

Biological transglycosylation of neamine by use of Pan's system was reported by Endo and Perlman.⁷⁰ The product was shown to be 5-*O*-D-glucosylneamine. Kanamycin A was also found to be an acceptor in the transglycosylation system.⁷¹

(65) M. J. Cron, D. L. Evans, F. M. Palermiti, D. F. Whitehead, I. R. Hooper, P. Chu, and R. U. Lemieux, *J. Amer. Chem. Soc.*, **80**, 4741 (1958).

(66) R. U. Lemieux, T. L. Nagabhushan, K. J. Clemetson, and L. C. N. Tucker, *Can. J. Chem.*, **51**, 53 (1973).

(67) A. Hasegawa, D. Nishimura, and M. Nakajima, *Agr. Biol. Chem.* (Tokyo), **36**, 1043 (1972).

(68) T. Ito, M. Nishio, and H. Ogawa, *J. Antibiot.* (Tokyo), **A17**, 189 (1964).

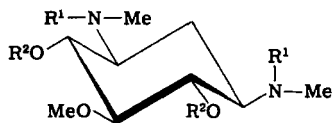
(69) M. Murase, *J. Antibiot.* (Tokyo), **A14**, 367 (1961).

(70) T. Endo and D. Perlman, *J. Antibiot.* (Tokyo), **25**, 681 (1972).

(71) T. Endo and D. Perlman, *J. Antibiot.* (Tokyo), **25**, 751 (1972).

b. Tobramycin.—Stark and colleagues⁷² reported the isolation of tobramycin (previously referred to as nebramycin factor 6), which was separated from an antibiotic complex, nebramycin, produced by *Streptomyces tenebrarius*. Among at least seven antibacterial factors in the complex, factors 2, 4, 5, and 6 were isolated.⁷³ Tobramycin has a broad spectrum of activity against a variety of bacteria, including *Pseudomonas* and *Proteus*.

The structure of tobramycin was reported by Koch and Rhoades.⁷⁴ Acid hydrolysis followed by treatment with methanolic hydrogen chloride gave methyl 3-amino-3-deoxy- β -glucoside and a new pseudodisaccharide named nebramine. Methanolysis of penta-*N*-acetyltobramycin gave methyl 3-amino-3-deoxy- β -glucoside and a mixture of partially deacetylated compounds, which gave, by *N*-acetylation, 1,3-di-*N*-acetyl-2-deoxystreptamine and the methyl α - β -glycoside of the *N,N'*-diacetyl derivative of nebramine, a new amino sugar. Hydrolysis of exhaustively *N,O*-methylated penta-*N*-acetyltobramycin, followed by reacetylation, afforded 1,3-di-*N*-acetyl-4,6-di-*O*-acetyl-1,3-di-*N*-methyl-5-*O*-methyl-2-deoxystreptamine (30). Longer hydrolysis afforded 1,3-di-*N*-methyl-2-deoxystreptamine (31), and further acetylation of the mother liquor afforded 1,3-di-*N*-acetyl-1,3-di-*N*-methyl-5-*O*-methyl-2-deoxystreptamine (32),



30 $R^1 = R^2 = \text{Ac}$

31 $R^1 = R^2 = \text{H}$

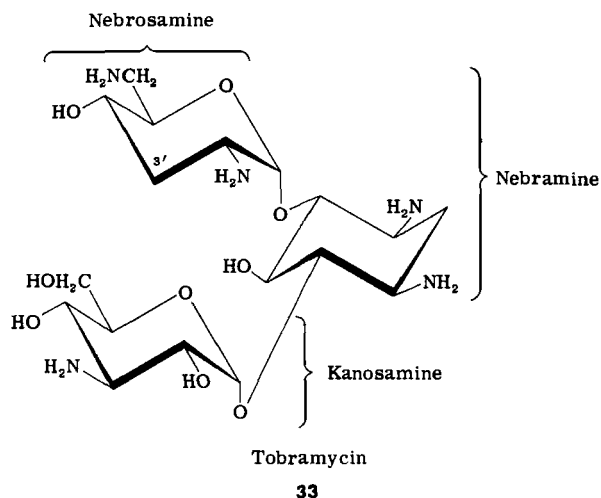
32 $R^1 = \text{Ac}, R^2 = \text{H}$

which was inert to periodate oxidation. On the basis of these results and of detailed, n.m.r.-spectral studies of tobramycin (by comparison with those of nebramine and the methyl α - β -glycoside of *N,N'*-diacetylnebramine), the absolute structure 33 was assigned to tobramycin. Tobramycin is 3'-deoxykanamycin B, and nebramine is 3'-deoxyneamine. The total synthesis of tobramycin has been accomplished (see p. 162).

(72) W. M. Stark, M. M. Hoehn, and N. G. Knox, *Antimicrob. Agents Chemother.*, 314 (1967).

(73) R. Q. Thompson and E. A. Presti, *Antimicrob. Agents Chemother.*, 332 (1967).

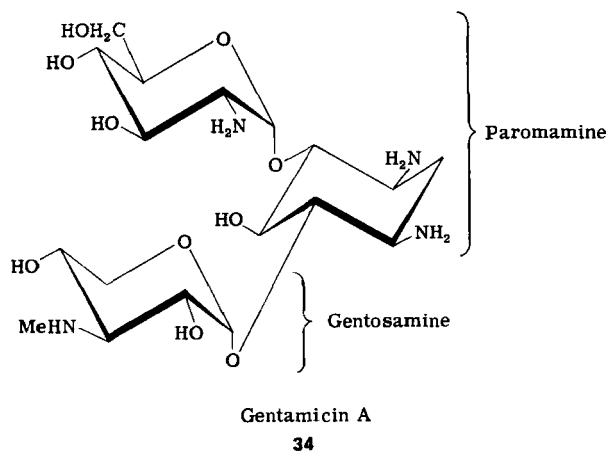
(74) K. F. Koch and J. A. Rhoades, *Antimicrob. Agents Chemother.*, 309 (1970).



4. Gentamicins and Sisomicin

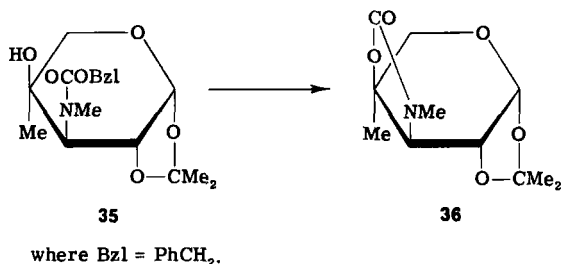
a. **Gentamicins.**—Weinstein and colleagues⁷⁵ reported the isolation of gentamicins, an antibiotic complex produced by *Micromonospora purpurea* and *Micromonospora echinospora*. The complex includes gentamicins A, C₁, C_{1a}, and C₂, and it has a broad spectrum of antibacterial activity against a variety of bacteria, including *Pseudomonas* and *Proteus*.

The structure of gentamicin A (34) was elucidated by Maehr and Schaffner.⁷⁶ Methanolysis of gentamicin A afforded paromamine and a new sugar, methyl gentosaminide, which was identified, by n.m.r.-spectral



studies, as methyl 3-deoxy-3-(methylamino)-D-xylopyranoside, and was synthesized from D-arabinose⁷⁶ or 3-amino-3-deoxy-D-glucose.⁷⁷ The glycosidic linkage between gentosamine and 2-deoxystreptamine was assigned on the basis of n.m.r.-spectral studies of tetra-*N*-acetylgentamicin A.⁷⁸

The gentamicins C₁, C₂, and C_{1a} are composed of three components, namely, 2-deoxystreptamine, an aminopentose named garosamine, and a hexose derivative (purpurosamine A, B, or C, respectively). Therefore, it is only the third component that differentiates between the three gentamicins C. Methanolysis⁷⁹ of the gentamicins C gave methyl garosaminide and pseudodisaccharides (named gentamines), which are glycosides of 2-deoxystreptamine and purpurosamine A, B, and C for the gentamicins C₁, C₂, and C_{1a}, respectively. Garosamine was the only natural, branched-chain amino sugar to have been found up to that time. The structure of garosamine was established⁷⁹ by mass and n.m.r. spectral studies, and by measurements of the change of molecular rotation upon complexing in Cupra B solution.²⁶ The structure assigned was supported by the formation of an oxazolidine derivative (36) from the protected derivative 35 by treatment with an anion-exchange resin.⁸⁰



Mercaptolysis of the penta-*N*-acetylgentamicins C gave *N,N'*-diacetyl-2-deoxystreptamine and the diethyl dithioacetal derivative of *N,N'*-diacetyl-

(75) M. J. Weinstein, C. M. Luedemann, E. M. Oden, G. H. Wagen, J. P. Rosselet, J. A. Marquez, C. T. Coniglio, W. Charney, H. L. Herzog, and J. Black, *J. Med. Chem.*, **6**, 463 (1963).

(76) H. Maehr and C. P. Schaffner, *J. Amer. Chem. Soc.*, **92**, 1697 (1970).

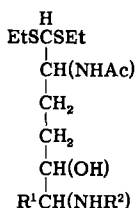
(77) W. Meyer zu Reckendorf and E. Bischof, *Chem. Ber.*, **105**, 2546 (1972); *Tetrahedron Lett.*, 2475 (1970).

(78) D. J. Cooper, M. D. Yudis, H. M. Marigliano, and T. Traubel, *J. Chem. Soc. (C)*, 2876 (1971).

(79) D. J. Cooper, M. D. Yudis, R. D. Guthrie, and A. M. Prior, *J. Chem. Soc. (C)*, 960 (1971).

(80) W. Meyer zu Reckendorf and E. Bischof, *Angew. Chem.*, **83**, 729 (1971).

purpurosamine A, B, or C. Mass.⁸¹ and n.m.r.-spectral studies confirmed⁷⁸ that the structures of the thioacetals are those shown in formulas 37 to 39. Consequently, the purpurosamines A, B, and C were deduced to be



37 (From Gentamicin C₁: R¹ = R² = Me)

38 (From Gentamicin C₂: R¹ = Me, R² = Ac)

39 (From Gentamicin C_{1a}: R¹ = H, R² = Ac)

members of the 2,6-diamino-2,3,4,6-tetradeoxyhexose family, a new class of natural monosaccharides.⁷⁸ Guthrie and G. J. Williams⁸² synthesized the ethyl glycoside of 2,6-diacetamido-2,3,4,6-tetradeoxy-D-*threo*-hexose (D-epipurpurosamine), and established that purpurosamine C has the D-*erythro* configuration. N.m.r.-spectral and optical rotatory studies on purpurosamine glycosides also showed that they belong to the D-series.⁸³ A methyl glycoside (185, see p. 168) which corresponds to that of purpurosamine C has been synthesized.

Exhaustive methylation of N-acetylgentamicins C₁ and C₂ gave the same permethylated compound, and methylation of N-acetylgentamicin C_{1a} gave a different permethylated compound. Hydrolysis of the two methylated compounds gave 1,3-di-N-acetyl-1,3-di-N-methyl-5-O-methyl-2-deoxystreptamine (32), which is optically inactive.⁸⁴ Moreover, the determination of circular dichroism of the cuprammonium complexes of the tetra-N-acetylgentamines indicated a negative dihedral angle for the diol grouping,⁸⁵ showing the attachment of purpurosamine at O-4 of 2-deoxystreptamine. The structures of gentamicins C₁, C₂, and C_{1a} were depicted⁸⁶ as shown in formulas 40, 41, and 42, respectively.

(81) D. C. DeJongh and S. Hanessian, *J. Amer. Chem. Soc.*, **87**, 3744 (1965).

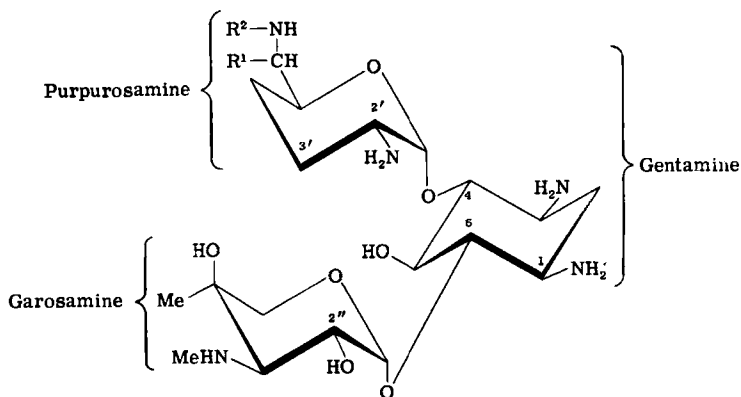
(82) R. D. Guthrie and G. J. Williams, *J. Chem. Soc. Perkin I*, 2619 (1972).

(83) P. J. L. Daniels, personal communication, cited in Ref. 82.

(84) D. J. Cooper, P. J. L. Daniels, M. D. Yudis, H. M. Marigliano, R. D. Guthrie, and S. T. K. Bukhari, *J. Chem. Soc. (C)*, 3126 (1971).

(85) S. T. K. Bukhari, R. D. Guthrie, A. I. Scott, and A. D. Wrixon, *Tetrahedron*, **26**, 3653 (1970).

(86) D. J. Cooper, *Pure Appl. Chem.*, **28**, 455 (1971).



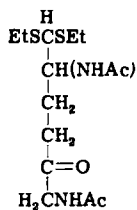
40 Gentamicin C₁: R¹ = R² = Me

41 Gentamicin C₂: R¹ = Me, R² = H

42 Gentamicin C_{1a}: R¹ = R² = H

b. Sisomicin.—Weinstein and colleagues⁸⁷ reported the isolation of sisomicin (formerly referred to as rickamicin), which is produced by *Micromonospora inyoensis*. Sisomicin was found to be active against various strains of bacteria, and effective against *Rickettsia akari* infection of mice.

Methyl garosaminide was isolated from the methanolizate of sisomicin.⁸⁸ Mercaptolysis of penta-*N*-acetylsisomicin gave 1,3-di-*N*-acetyl-2-deoxystreptamine and a diethyl dithioacetal (43) containing a ketone



43

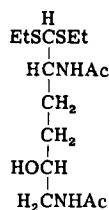
group. Its structure, established by n.m.r.-spectral studies, indicated the presence of a 2,6-diamino-2,3,4,6-tetradeoxy-D-glycero-hex-4-enosyl group (that of a dehydro-purpurosamine) in sisomicin. High-resolution, mass-

(87) M. J. Weinstein, J. A. Marquez, R. T. Testa, G. H. Wagman, E. M. Oden, and J. A. Waitz, *J. Antibiot.* (Tokyo), **23**, 551 (1970).

(88) D. J. Cooper, R. S. Jaret, and H. Reimann, *Chem. Commun.*, 285 (1971).

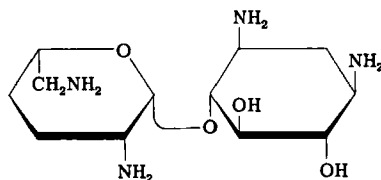
spectral studies of sisomicin also enabled location of the site of unsaturation.

Catalytic hydrogenation⁸⁹ of sisomicin gave dihydrosisomicin, which is isomeric with gentamicin C_{1a} (42). Comparison of the n.m.r. spectrum of the dihydrosisomicin with that of gentamicin C_{1a} indicated that, upon hydrogenation, an inversion of the unsaturated sugar ring occurs, giving an axially attached, anomeric proton in the dihydrosisomicin. Mercaptolysis of penta-*N*-acetyldihydrosisomicin, followed by reacetylation, gave the diethyl dithioacetal (44) of 2,6-diacetamido-2,3,4,6-tetradeoxy-*L*-*threo*-



44

hexose. Its stereochemistry was confirmed by n.m.r.-spectral comparison with the synthetic *D*-*threo* enantiomer.⁸² Methanolysis of dihydrosisomicin gave a pseudodisaccharide (45). The c.d. spectrum of its cuprammonium complex indicated that the pseudodisaccharide is a 4-*O*-glycosyl-2-deoxystreptamine.

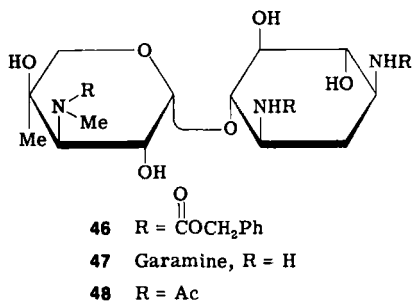


45

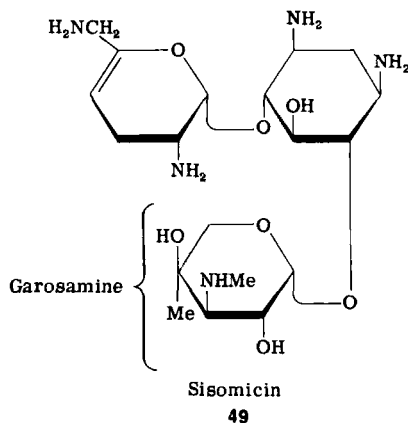
Penta-*N*-(benzyloxycarbonyl)sisomicin⁹⁰ was found to be highly labile towards even mildly acidic reagents, and to give *N*-(benzyloxycarbonyl)-garamine (46) which, on hydrogenation, afforded a pseudodisaccharide named garamine (47). The c.d. spectrum of the cuprammonium complex of *N*-acetylgaramine (48) indicated that the garosamine residue is linked at O-6 of 2-deoxystreptamine. Further evidence was afforded by the

(89) H. Reimann, R. S. Jaret, and D. J. Cooper, *Chem. Commun.*, 924 (1971).

(90) M. Kugelman, A. K. Mallams, and H. F. Vernay, *J. Antibiot. (Tokyo)*, **26**, 394 (1973).



isolation of 1,3-di-*N*-acetyl-1,3-di-*N*-methyl-5-*O*-methyl-2-deoxystreptamine (32, see p. 124) from the hydrolyzate of methylated penta-*N*-acetylsisomicin. The structure of sisomicin was depicted⁹⁰ by formula 49.



5. Ribostamycin and Butirosins (and Bu-1709 E1 and E2)

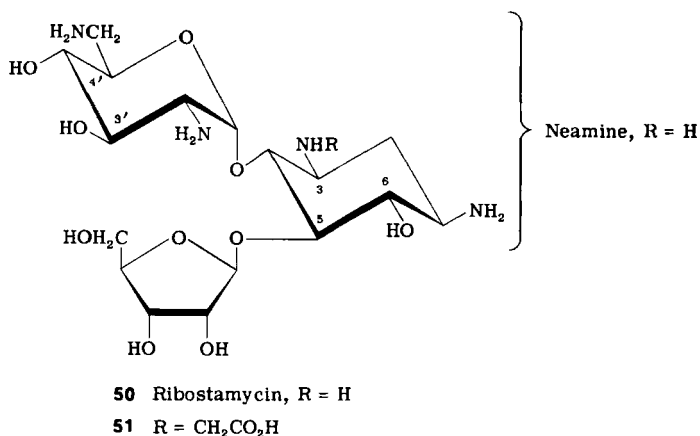
a. **Ribostamycin.**—Shomura and colleagues⁹¹ isolated this antibiotic (formerly called SF-733), which is produced by *Streptomyces ribosidificus*, and is a broad-spectrum antibiotic. Its structure (50) was reported by Akita and colleagues.⁹² On methanolysis, ribostamycin gives neamine (83, see p. 142) and a methyl *D*-riboside. The furanoid nature of the *D*-ribose moiety was shown by the results of periodate oxidation of tetra-*N*-acetylribostamycin. Evidence for the position of attachment of the *D*-ribose residue to 2-deoxystreptamine was afforded by the

(91) T. Shomura, N. Ezaki, T. Tsuruoka, T. Niwa, E. Akita, and T. Niida, *J. Antibiot.* (Tokyo), **23**, 155 (1970).

(92) E. Akita, T. Tsuruoka, N. Ezaki, and T. Niida, *J. Antibiot.* (Tokyo), **23**, 173 (1970).

isolation of 1,3-di-*N*-acetyl-6-*O*-methyl-2-deoxystreptamine (**13**, see p. 117) from the acid hydrolyzate of methylated tetra-*N*-acetylribostamycin. The β -D-linkage of the D-ribosyl group was arrived at from comparison of the n.m.r. spectrum of tetra-*N*-acetylribostamycin with those of the neomycins.⁴⁵ The total synthesis of ribostamycin has been accomplished (see p. 162).

Fermentation of ribostamycin was modified⁹³ to yield 3-*N*-(carboxymethyl)ribostamycin (**51**). The position of the carboxymethyl group was



ascertained by ¹³C n.m.r.-spectral studies on ribostamycin and related compounds.⁹⁴

b. Butirosins.—Woo and colleagues⁹⁵ isolated this antibiotic complex from the culture broth of *Bacillus circulans*. (Butirosin was formerly called ambutyrosin.) The antibiotic complex was resolved by chromatography on Dowex-1 X-1 or X-2 in the borate form; butirosin A was found to be the major component.

Mild hydrolysis of butirosin A and B with acid slowly liberated D-xylose and D-ribose, respectively. Hydrolysis with concentrated acid gave⁹⁶ neamine, neosamine C, 2-deoxystreptamine, and a novel amino acid, 4-amino-3,4-dideoxy-L-glycero-tetronic acid [(*S*)-(–)-4-amino-2-hydroxybutyric acid] (**53**). Treatment of the methyl ester of this amino

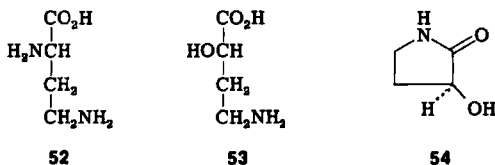
(93) M. Kojima, S. Inouye, and T. Niida, *J. Antibiot.* (Tokyo), **26**, 246 (1973).

(94) S. Omoto, S. Inouye, M. Kojima, and T. Niida, *J. Antibiot.* (Tokyo), **26**, 717 (1973).

(95) P. W. K. Woo, H. W. Dion, G. L. Coffey, S. A. Fusari, and G. Demetra, Ger. Pat. 1,914,527 (Oct. 9, 1969); *Chem. Abstr.*, **72**, 41,742 (1970); U. S. Pat. 3,541,078 (Nov. 17, 1970).

(96) P. W. K. Woo, H. W. Dion, and Q. R. Bartz, *Tetrahedron Lett.*, 2617 (1971).

acid with ammonia in methanol gave the lactam **54**. The amino acid was synthesized through partial deamination of 2,4-diamino-2,3,4-trideoxy-L-*glycero*-tetronic acid [L-(+)-2,4-diaminobutyric acid] (**52**) with sodium nitrite.



Mass-spectral studies⁹⁷ of the poly-*N*-acetyl-poly-*O*-(trimethylsilyl) derivatives of the butirosins A and B, with the aid of a deuterioacetyl analog, showed that three of the structural units, the amino acid, neosamine C, and a pentose, are individually attached, as the amide and the glycosides, to the fourth unit, 2-deoxystreptamine. Methylation and methanolysis studies⁹⁸ of tetra-*N*-acetylbutirosin A revealed the linkage position and ring size of the *D*-xylose moiety in butirosin A. The isolation of methyl 2,3,5-tri-*O*-methyl-*D*-xylofuranoside showed that the *D*-xylose is present in the furanoid form. Acid hydrolysis of the methanolysis product remaining gave, among other products, 6-*O*-methyl-2-deoxystreptamine and 4-amino-3,4-dideoxy-2-*O*-methyl-L-*glycero*-tetronic acid. The isolation of the former indicated that the *D*-xylofuranosyl group is attached to O-5 of 2-deoxystreptamine, and the isolation of the latter indicated that the 2-hydroxyl group in the amino acid is unsubstituted. The n.m.r. spectra of the tetra-*N*-acetylbutirosins A and B indicated the presence of β -*D*-xylofuranosyl and β -*D*-ribofuranosyl linkages, respectively. Periodate-oxidation studies of the butirosins A and B revealed the position of linkage of the amino acid. Thus, the structures of the butirosins A and B were established by Woo and colleagues⁹⁸ as being those depicted in formulas **55** and **56**, respectively. The total synthesis of butirosin B has been accomplished (see p. 163).

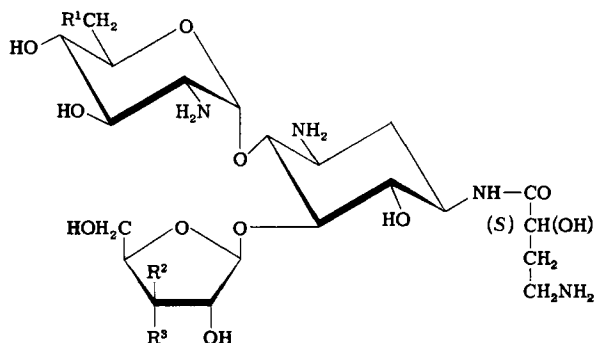
1-*N*-[(*S*)-4-Amino-2-hydroxybutyryl]neamine (**208**, see p. 175) was obtained from butirosins A and B by controlled methanolysis, and found to have antibacterial activity.⁹⁹ From another butirosin complex, two analogs, Bu-1709 E₁ (**57**) and E₂ (**58**), containing a paromamine moiety instead of the neamine moiety in the butirosins, were isolated.¹⁰⁰

(97) P. W. K. Woo, *Tetrahedron Lett.*, 2621 (1971).

(98) P. W. K. Woo, H. W. Dion, and Q. R. Bartz, *Tetrahedron Lett.*, 2625 (1971).

(99) H. Tsukiura, K. Fujisawa, M. Konishi, K. Saito, K. Numata, H. Ishikawa, T. Miyaki, K. Tomita, and H. Kawaguchi, *J. Antibiot.* (Tokyo), **26**, 351 (1973).

(100) H. Tsukiura, K. Saito, S. Kobaru, M. Konishi, and H. Kawaguchi, *J. Antibiot.* (Tokyo), **26**, 386 (1973).



55 Butirosin A, $R^1 = \text{NH}_2$, $R^2 = \text{OH}$, $R^3 = \text{H}$

56 Butirosin B, $R^1 = \text{NH}_2$, $R^2 = \text{H}$, $R^3 = \text{OH}$

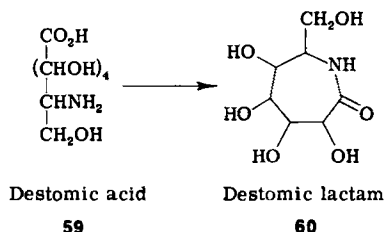
57 Bu-1709 E₁, $R^1 = R^2 = \text{OH}$, $R^3 = \text{H}$

58 Bu-1709 E₂, $R^1 = \text{OH}$, $R^2 = \text{H}$, $R^3 = \text{OH}$

6. Hygromycin B and Destomycin A (and A-396-I and SS-56C)

a. Hygromycin B and Destomycin A.—Hygromycin B, reported by Mann and Bromer¹⁰¹ in 1958, and destomycin A, reported by Kondo and colleagues¹⁰² in 1965, are closely related antibiotics, useful against animal ascariasis. The former is produced by *Streptomyces hygroscopicus*, and the latter, by *Streptomyces rimofaciens*. Both antibiotics consist of an *N*-methyl-2-deoxystreptamine residue, a D-talose residue, and a destomic acid (59) residue. It is the first component that differentiates between the two antibiotics.

Complete hydrolysis of destomycin A yielded the aforementioned three components and destomic lactam (60). Mild hydrolysis with acid yielded



N-methyl-5-*O*-β-D-talopyranosyl-2-deoxystreptamine.¹⁰³ Methylation and

(101) R. L. Mann and W. W. Bromer, *J. Amer. Chem. Soc.*, **80**, 2714 (1958).

(102) S. Kondo, M. Sezaki, M. Koike, M. Shimura, E. Akita, K. Sato, and T. Hara, *J. Antibiot.* (Tokyo), **A18**, 38 (1965).

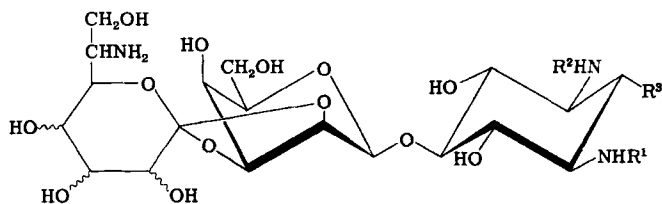
(103) S. Kondo, M. Sezaki, M. Koike, and E. Akita, *J. Antibiot.* (Tokyo), **A18**, 192 (1965).

hydrolysis studies of tri-*N*-acetyldestomycin A revealed the unusual, ortho ester type of attachment of the 2- and 3-hydroxyl groups of the *D*-talosyl residue to the destomic acid residue, and Kondo and coworkers¹⁰⁴ first elucidated the gross structure of destomycin A. The *N*-methyl-2-deoxystreptamine from destomycin A is levorotatory and that from hygromycin B is dextrorotatory. O.r.d.-spectral studies differentiated between the two aminocyclitols.¹⁰⁵ ¹³C n.m.r.-spectral studies by Roberts and coworkers¹⁰⁵ permitted assignments of chemical shifts to resonances of all of the carbon atoms and *N*-methyl in the hyosamine (3-*N*-methyl-2-deoxystreptamine) moiety, and the ortho ester carbon atom of the destomic acid, which showed a chemical shift similar to that of triethyl orthoformate. *DL*-Hyosamine^{106,107} and *L*-hyosamine¹⁰⁸ have been synthesized.

b. A-396-I and SS-56C.—Shoji and colleagues¹⁰⁹ isolated the antibiotic A-396-I from a culture of *Streptovercillium eurocidicus*. Hydrolysis¹¹⁰ afforded 2-deoxystreptamine, in addition to *D*-talose and destomic acid. The n.m.r. spectra of A-396-I and hygromycin B are similar, except that *N*-CH₃ observed in the spectrum of the latter is not observed in that of the former. The mass spectrum of peracetylated A-396-I is similar to that of hygromycin B, with a difference of mass number 14 (CH₂).

Another related antibiotic, SS-56C, was reported by Inouye and colleagues.¹¹¹ This antibiotic contains a streptamine residue instead of the 2-deoxystreptamine residue in A-396-I. From the culture broth of *Streptomyces eurocidicus*, *D*-mannosyl- and *D*-talosyl-2-deoxystreptamine were also isolated. In the structural studies, the mass spectra of their *N*-salicylidene derivatives were used.¹⁰

Thus, it is possible to describe the structures of destomycin A, hygromycin B, A-396-I, and SS-56C by formulas 61 to 64.



- 61** Destomycin A, $R^1 = \text{Me}$, $R^2 = \text{H}$, $R^3 = \text{H}$
62 Hygromycin B, $R^1 = \text{H}$, $R^2 = \text{Me}$, $R^3 = \text{H}$
63 A-396-I, $R^1 = R^2 = R^3 = \text{H}$
64 SS-56C, $R^1 = R^2 = \text{H}$, $R^3 = \text{OH}$

(104) S. Kondo, E. Akita, and M. Koike, *J. Antibiot.* (Tokyo), **A19**, 139 (1966).

(105) N. Neuss, K. F. Koch, B. B. Molloy, W. Day, L. L. Huckstep, D. E. Dorman, and J. D. Roberts, *Helv. Chim. Acta*, **53**, 2314 (1970).

7. Miscellaneous Chemical Types: Kasugamycin, Spectinomycin, α,α -Trehalosamine and α -D-Mannosyl 2-Amino-2-deoxy- α -D-glucoside, Validamycins, and Apramycin

a. **Kasugamycin.**—H. Umezawa and coworkers¹¹² isolated this antibiotic, and reported^{113,114} its unique structure (65). Kasugamycin is produced by *Streptomyces kasugaensis*, and is useful as a powerful inhibitor of *Pericaria oryzae* in rice plants.

Acid hydrolysis¹¹⁵ of kasugamycin gave (+)-inositol. Methanolysis gave a methyl glycoside, in addition to the inositol. Alkaline hydrolysis of the methyl glycoside gave oxalate, ammonia, and a novel aminoglycoside, methyl kasugaminide, which was found to be resistant to periodate oxidation. Oxidation¹¹⁶ of *N,N'*-diacetylkasugamine with bromine water gave a δ -lactone, indicating that the kasugamine moiety has the pyranoid form. N.m.r.-spectral study of the methyl kasugaminide showed that kasugamine is 2,4-diamino-2,3,4,6-tetra-deoxy-D-arabino-hexose.

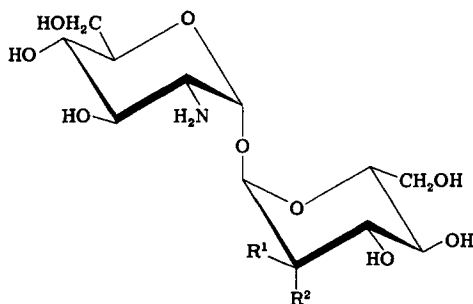
Alkaline hydrolysis¹¹⁷ of kasugamycin (65) with barium hydroxide afforded kasugamycinic acid (66) and kasuganobiosamine (67), which are devoid of antibiotic activity. The α -D anomeric configuration in kasugamycin was shown by n.m.r.-spectral data.¹¹⁷ Kasugamycinic acid gave kasuganobiosamine and oxalic acid by alkaline hydrolysis, and (+)-inositol by acid hydrolysis. Mono-*N*-acetylation of kasugamycinic

-
- (106) M. Nakajima, A. Hasegawa, and N. Kurihara, *Ann.*, **689**, 235 (1965).
(107) T. Suami, S. Ogawa, and H. Sano, *Bull. Chem. Soc. Jap.*, **43**, 1843 (1970).
(108) N. Kurihara, K. Hayashi, and M. Nakajima, *Agr. Biol. Chem.* (Tokyo), **33**, 256 (1969).
(109) J. Shoji, S. Kozuki, M. Mayama, Y. Kawamura, and K. Matsumoto, *J. Antibiot.* (Tokyo), **23**, 291 (1970).
(110) J. Shoji and Y. Nakagawa, *J. Antibiot.* (Tokyo), **23**, 569 (1970).
(111) S. Inouye, T. Shomura, H. Watanabe, K. Totsugawa, and T. Niida, *J. Antibiot.* (Tokyo), **26**, 374 (1973).
(112) H. Umezawa, Y. Okami, T. Hashimoto, Y. Suhara, M. Hamada, and T. Takeuchi, *J. Antibiot.* (Tokyo), **A18**, 101 (1965).
(113) Y. Suhara, K. Maeda, H. Umezawa, and M. Ohno, *Tetrahedron Lett.*, 1239 (1966).
(114) Y. Suhara, K. Maeda, H. Umezawa, and M. Ohno, in "Deoxy Sugars," *Advan. Chem. Ser.*, No. 74, 15 (1968).
(115) Y. Suhara, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **A18**, 182 (1965).
(116) Y. Suhara, K. Maeda, H. Umezawa, and M. Ohno, *J. Antibiot.* (Tokyo), **A18**, 184 (1965).
(117) Y. Suhara, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **A18**, 187 (1965).

was ascertained by an X-ray crystallographic analysis of spectinomycin dihydrobromide pentahydrate by Cochran and coworkers.¹²¹ The B/C ring juncture is *cis*, with the hydroxyl group in an axial orientation with respect to ring B, and the stereochemistry of ring A is equivalent to that of the C-2 epimer of streptomycin. The absolute configuration of the tertiary carbon atom in the B/C ring junction was shown to be *S*, and that of the methyl-bearing carbon atom in ring C, to be *R*. In addition, it was found that the pentahydrate exists as a ketone hydrate, and is not present in the carbonyl form. The ketone hydrate form also preponderates in aqueous solution. The stability of the unusual ketone hydrate was considered to be due to the presence of the four electronegative oxygen atoms bonded to the B/C ring juncture. Actinamine has been synthesized.¹²²⁻¹²⁷

c. α,α -Trehalosamine and α -D-Mannosyl 2-Amino-2-deoxy- α -D-glucoside. —These antibiotics are nonreducing disaccharides. The chemistry of α,α -trehalosamine (69) has been reviewed,¹²⁸ and the compound has been synthesized (see p. 153).

Yonehara and coworkers¹²⁹ isolated α -D-mannosyl 2-amino-2-deoxy- α -D-glucoside (70), which is coproduced with α,α -trehalosamine by *Strepto-*



69 α,α -Trehalosamine, $R^1 = OH$, $R^2 = H$

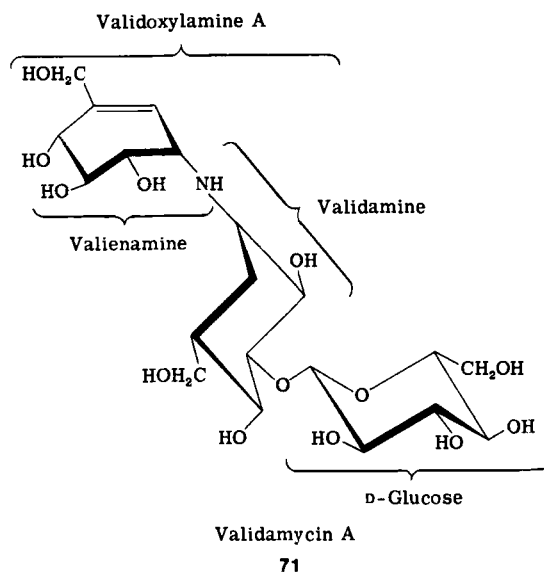
70 α -D-Mannosyl 2-amino-2-deoxy- α -D-glucoside, $R^1 = H$, $R^2 = OH$

myces virginiae; it has antibacterial and antifungal properties.

- (121) T. G. Cochran, D. J. Abraham, and L. L. Martin, *J. Chem. Soc. Chem. Commun.*, 494 (1972).
- (122) M. Nakajima, N. Kurihara, A. Hasegawa, and T. Kurokawa, *Ann.*, **689**, 243 (1965).
- (123) F. W. Lichtenthaler, H. Leinert, and L. L. Martin, *Angew. Chem. Int. Ed. Engl.*, **6**, 254 (1967).
- (124) F. W. Lichtenthaler, H. Leinert, and T. Suami, *Chem. Ber.*, **100**, 2383 (1967).
- (125) S. Ogawa, T. Abe, H. Sano, K. Kotera, and T. Suami, *Bull. Chem. Soc. Jap.*, **40**, 2405 (1967).

d. **Validamycins.**—Iwasa and colleagues¹³⁰ isolated the validamycin complex produced by *Streptomyces hygroscopicus* var. *limoneus*. Validamycins A, B, C, D, E, and F were found, A being the main component. Validamycins were found to be effective against certain pathogenic fungi in plants in greenhouses, although they are inactive *in vitro*.

The structure of validamycin A (71) was reported by Horii and Kameda.¹³¹ In contrast to the other aminoglycoside antibiotics, all of the



validamycins contain a residue of a novel, unsaturated aminocyclitol named valienamine.¹³² Acid hydrolysis of validamycin A yields D-glucose and validoxylamine A; on hydrogenolysis, the latter gives validamine, validatol (72), and deoxyvalidatol (73). The n.m.r. spectrum of validoxylamine A indicated the presence of the partial structure
 $\text{HOCH}_2-\text{C}=\text{CH}-$, and, after hydrogenolysis, signals of the ring-

(126) T. Suami, S. Ogawa, S. Naito, and H. Sano, *J. Org. Chem.*, **33**, 2831 (1968).

(127) T. Suami and H. Sano, *Tetrahedron Lett.*, 2655 (1968).

(128) G. G. Birch, *Advan. Carbohydr. Chem.*, **18**, 219 (1963).

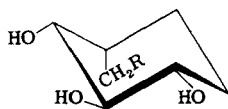
(129) M. Uramoto, N. Otake, and H. Yonehara, *J. Antibiot. (Tokyo)*, **A20**, 236 (1967).

(130) T. Iwasa, H. Yamamoto, and M. Shibata, *J. Antibiot. (Tokyo)*, **23**, 595 (1970).

(131) S. Horii and Y. Kameda, *J. Chem. Soc. Chem. Commun.*, 747 (1973).

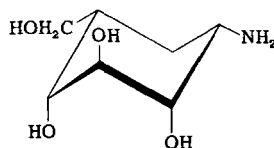
(132) S. Horii, T. Iwasa, and Y. Kameda, *J. Antibiot. (Tokyo)*, **24**, 57 (1971).

methylene protons and the tertiary ring-proton of validatol and deoxyvalidatol became evident. Interestingly, by use of a cell suspension of *Pseudomonas denitrificans*, validamycin A was biologically hydrolyzed to D-glucose, validamine, and valienamine. By the same degradation, dihydrovalidamycin A gave validamine and a new aminocyclitol named epivalidamine (74), but no valienamine. The n.m.r.-spectral and periodate-



72 Validatol, R = OH

73 Deoxyvalidatol, R = H

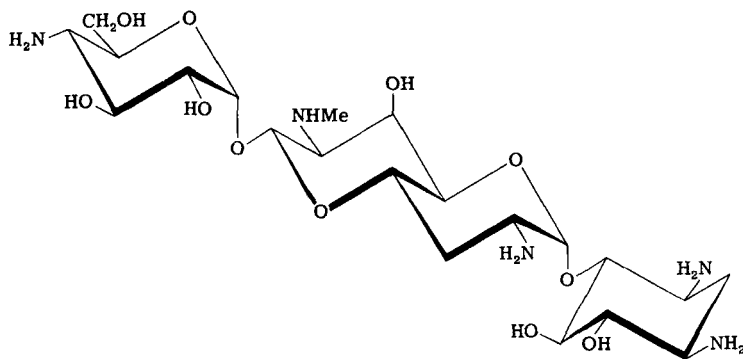


Epivalidamine

74

oxidation studies of these degradation products revealed their structures.^{133,134} Catalytic hydrogenation of validamycin A yielded dihydrovalidamycin A and monodeoxydihydrovalidamycin A, in addition to β -D-glucopyranosylvalidamine, validatol, and deoxyvalidatol.^{135,136} The isolation of validamine after periodate oxidation of the N-acetyl derivative of β -D-glucopyranosylvalidamine indicated the substitution at O-3 of validamine with a D-glucosyl group.¹³¹

e. Apramycin.—This broad-spectrum antibiotic, produced by *Streptomyces tenebrarius*, was reported by O'Connor and Lam¹³⁷ to have the structure depicted in formula 75. A unique feature in its structure is the



Apramycin

75

(133) S. Horii, Y. Kameda, and K. Kawahara, *J. Antibiot.* (Tokyo), **25**, 48 (1972).

(134) Y. Kameda and S. Horii, *J. Chem. Soc. Chem. Commun.*, 746 (1972).

presence of an octodiose that exists as a rigid, bicyclic system. A detailed examination of the antibiotic that involved degradation and n.m.r.- and mass-spectral studies led to the assignment of the absolute structure.

III. TOTAL SYNTHESIS OF AMINOGLYCOSIDE ANTIBIOTICS AND RELATED COMPOUNDS

For the purpose of reviewing the total synthesis of aminoglycoside antibiotics, it is convenient to divide them into two groups according to the number of monosaccharides, including pseudomonosaccharides, of which they are composed. These two groups are: aminoglycosides composed of two carbohydrate moieties, and those composed of three carbohydrate moieties. Chemical modification of aminoglycoside antibiotics will be described in Section IV (see p. 164).

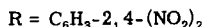
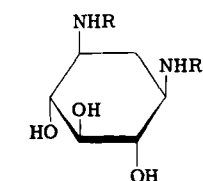
1. Total Synthesis of Aminoglycosides Composed of Two Carbohydrate Moieties

Paromamine, neamine, 4-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-2-deoxystreptomine, 5-*O*-(2-amino-2-deoxy- α -D-glucopyranosyl)-2-deoxystreptomine, kasugamycin, and α,α -trehalosamine have been synthesized. A common feature in the structures of these antibiotics is the presence of an α -D-glycosidic linkage. In the total syntheses, modified Koenigs-Knorr reactions, or addition of nitrosyl chloride¹³⁸⁻¹⁴¹ to an enose, were successfully used.

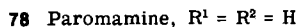
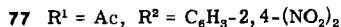
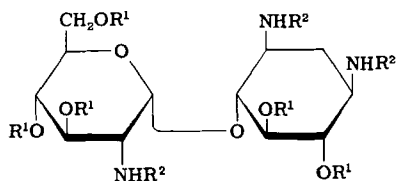
a. Synthesis of Paromamine.—Umezawa and Koto¹⁴² synthesized paromamine (78) in 1966, and this was the first synthesis of an amino-

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- (135) S. Horii, T. Iwasa, E. Mizuta, and Y. Kameda, *J. Antibiot.* (Tokyo), **24**, 59 (1971).
(136) K. Kamiya, Y. Wada, S. Horii, and M. Nishikawa, *J. Antibiot.* (Tokyo), **24**, 317 (1971).
(137) S. O'Connor and L. K. T. Lam, *Abstr. Papers Amer. Chem. Soc. Meeting*, **165**, MEDI 6 (1973).
(138) R. U. Lemieux, T. L. Nagabhushan, and I. K. O'Neill, *Tetrahedron Lett.*, 1909 (1964).
(139) W. J. Serfontein, J. H. Jordaan, and J. White, *Tetrahedron Lett.*, 1069 (1964).
(140) R. U. Lemieux, Y. Ito, K. James, and T. L. Nagabhushan, *Can. J. Chem.*, **51**, 7 (1973).
(141) R. U. Lemieux, K. James, and T. L. Nagabhushan, *Can. J. Chem.*, **51**, 42 (1973).
(142) S. Umezawa and S. Koto, *J. Antibiot.* (Tokyo), **A19**, 88 (1966); *Bull. Chem. Soc. Jap.*, **39**, 2014 (1966).

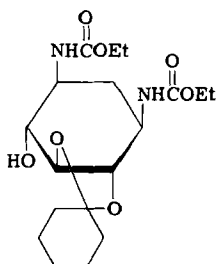
glycoside antibiotic. Condensation of 3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)- α -D-glucopyranosyl bromide^{143,144} with bis-*N,N'*-(2,4-dinitrophenyl)deoxystreptamine (76) in nitromethane in the presence of mercuric cyanide and bromide gave a mixture of glycosides, and subsequent acetylation followed by separation by preparative t.l.c. gave the masked paromamine 77 in a low yield. Hydrolysis with methanolic ammonia and treatment with Dowex-1 X-2 (OH⁻) gave paromamine (78).



76

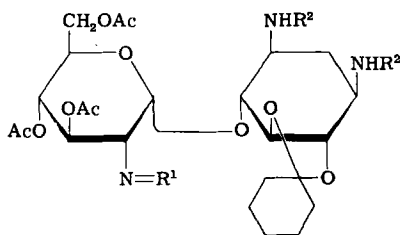


An improved synthesis of paromamine¹⁴⁵ was effected with a Schiff base and a more protected 2-deoxystreptamine. The cyclohexylidene derivative of 1,3-di-*N*-(ethoxycarbonyl)-2-deoxystreptamine (79, racemate) was condensed with 3,4,6-tri-*O*-acetyl-2-deoxy-2-(*p*-methoxybenzylideneamino)- α -D-glucopyranosyl bromide (see Refs. 174 and 175 on p. 153) to give two products (80 and 81), which, after removal of the protecting groups, furnished paromamine (78) and an isomer, 6-*O*-(2-amino-2-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine (82). The latter was devoid of antibacterial activity.



(Racemate)

79

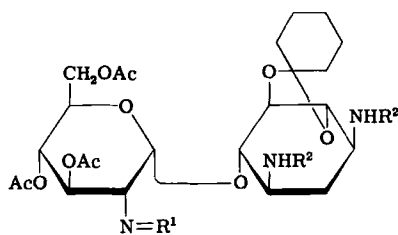


80

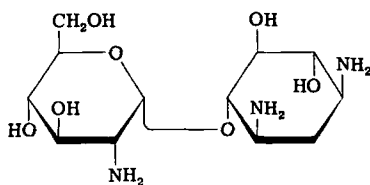
(143) P. F. Lloyd and M. Stacey, *Chem. Ind.* (London), 917 (1955).

(144) D. Horton, *J. Org. Chem.*, **29**, 1776 (1964).

(145) S. Umezawa, T. Miyazawa, and T. Tsuchiya, *J. Antibiot.* (Tokyo), **25**, 530 (1972).



81

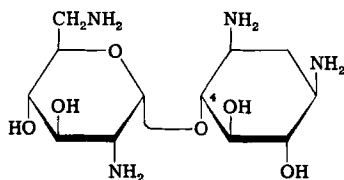


82

The synthesis of paromamine subsequently led to the synthesis of kanamycin C (see p. 159).

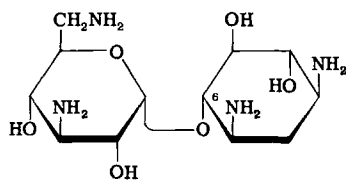
b. Synthesis of Neamine (Neomycin A) and Related Compounds.—Umezawa and coworkers¹⁴⁶ synthesized neamine from paromamine. Treatment of peracetylated paromamine with methanolic methoxide gave tri-*N*-acetylparomamine. Selective *p*-toluenesulfonylation of its primary hydroxyl group, followed by replacement of the *p*-tolylsulfonyloxy group by an azido group, amination by catalytic reduction, and finally, *N*-deacetylation with hydrazine, afforded neamine (83).

A similar sequence of reactions¹⁴⁷ was applied to 6-*O*-(3-amino-3-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine (obtained by partial hydrolysis of the kanamycins) to yield a structural isomer of neamine, namely, 6-*O*-(3,6-diamino-3,6-dideoxy- α -D-glucopyranosyl)-2-deoxystreptamine (84), which was devoid of activity.



Neamine

83



84

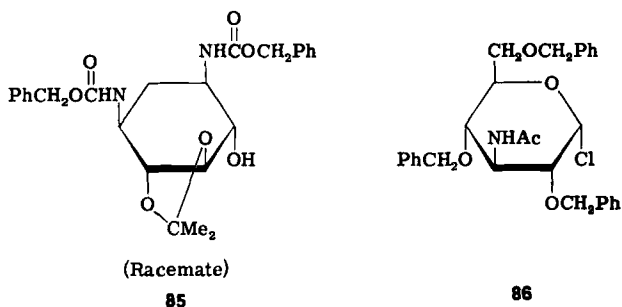
c. Synthesis of 4-*O*-(6-Amino-6-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine, 6-*O*-(3-Amino-3-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine,

(146) S. Umezawa, K. Tatsuta, T. Tsuchiya, and E. Kitazawa, *J. Antibiot.* (Tokyo), **A20**, 53 (1967).

(147) K. Tatsuta, K. Kitazawa, and S. Umezawa, *Bull. Chem. Soc. Jap.*, **40**, 2371 (1967).

mine, and Related Compounds.—6-*O*-(3-Amino-3-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine is the glycoside component common to the kanamycins, and was obtained from kanamycin A by partial hydrolysis.⁶⁰ 4-*O*-(6-Amino-6-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine was also obtained⁶¹ by partial hydrolysis of kanamycin A. The latter has antibacterial activity, whereas the former has not. Syntheses of both glycosides were studied as approaches to the synthesis of the kanamycins.

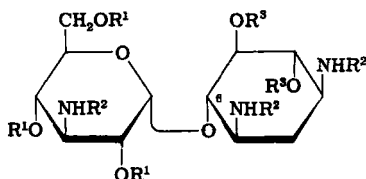
6-*O*-(3-Amino-3-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine (**88**) was synthesized by Umezawa and coworkers¹⁴⁸ and by Nakajima and coworkers.¹⁴⁹ In both of the syntheses, the isopropylidene derivative (**85**) of 1,3-di-*N*-(benzyloxycarbonyl)-2-deoxystreptamine was prepared as a key intermediate. (It is noteworthy that protection of the *trans* hydroxyl groups was achieved by an acetal-exchange reaction.^{150,151}) Also, the tri-*O*-benzylglycosyl chloride¹⁵² (**86**) of 3-acetamido-3-deoxy-D-glucose¹⁵³



was prepared. (In regard to the benzyl sugars, Baddiley and colleagues¹⁵⁴ had reported that tetra-*O*-benzyl-D-glucopyranosyl chloride provides a route to the preparation of the α -D-glucoside.) Condensation¹⁴⁸ of **85** with **86** was conducted in anhydrous benzene-1,4-dioxane in the presence of mercuric cyanide and Drierite. Removal of the protecting groups fol-

- (148) S. Koto, K. Tatsuta, E. Kitazawa, and S. Umezawa, *Bull. Chem. Soc. Jap.*, **41**, 2769 (1968).
- (149) A. Hasegawa, N. Kurihara, D. Nishimura, and M. Nakajima, *Agr. Biol. Chem. (Tokyo)*, **32**, 1123 (1968).
- (150) M. E. Evans, F. W. Parrish, and L. Long, Jr., *Carbohydr. Res.*, **3**, 453 (1967).
- (151) S. J. Angyal and R. M. Hoskinson, *J. Chem. Soc.*, 2985 (1962).
- (152) S. Koto, T. Tsumura, Y. Kato, and S. Umezawa, *Bull. Chem. Soc. Jap.*, **41**, 2765 (1968).
- (153) H. H. Baer, *J. Amer. Chem. Soc.*, **83**, 1882 (1961).
- (154) P. W. Austin, F. E. Hardy, J. G. Buchanan, and J. Baddiley, *J. Chem. Soc.*, 2128 (1964).

lowed by *N*-(2,4-dinitrophenyl)ation, reacylation, and chromatography afforded the condensation product **87** in 12% yield. Hydrolysis with methanolic ammonia followed by treatment with Dowex-1 X-2 (OH⁻) afforded **88**.



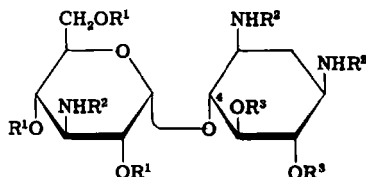
87 $R^1 = R^3 = \text{Ac}$, $R^2 = \text{C}_6\text{H}_3-2,4-(\text{NO}_2)_2$

88 $R^1 = R^3 = R^2 = \text{H}$

89 $R^1 = \text{CH}_2\text{Ph}$, $R^2 = \text{COCH}_2\text{Ph}$, $R^3 = \text{H}$

90 $R^1 = R^3 = \text{H}$, $R^2 = \text{Ac}$

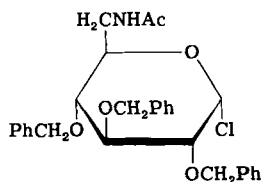
In the alternative synthesis,¹⁴⁹ the condensation of **85** with **86** was performed in chloroform-1,4-dioxane in the presence of silver carbonate, silver perchlorate, and Drierite. Deacetonation followed by chromatography afforded the protected 6-glycoside (**89**) and the 4-isomer (**91**) in almost the same amounts; these afforded the tri-*N*-acetyl derivatives **90** and **92**, respectively.



91 $R^1 = \text{CH}_2\text{Ph}$, $R^2 = \text{COCH}_2\text{Ph}$, $R^3 = \text{H}$

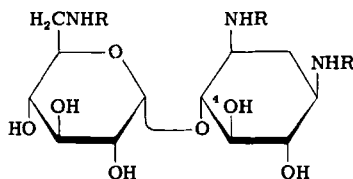
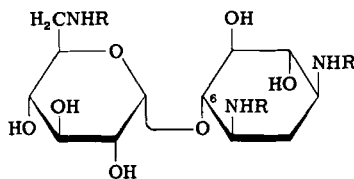
92 $R^1 = R^3 = \text{H}$, $R^2 = \text{Ac}$

The tri-*N*-acetyl derivative (**94**) of 4-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine (**95**) was synthesized by Nakajima and coworkers.¹⁴⁶ Condensation of 6-acetamido-2,3,4-tri-*O*-benzyl-6-deoxy- α -D-glucopyranosyl chloride¹⁵⁵ (**93**) with the 2-deoxystreptamine deriv-



93

ative **85** gave a mixture of the 4- and 6-glycosides, which then afforded the tri-*N*-acetyl glycosides **94** and **96**.

**94** R = Ac**95** R = H**96** R = Ac**97** R = H

Another synthesis of **95** (and **97**) reported by Umezawa and co-workers¹⁵⁸ involved the condensation of 6-azido-2,3,4-tri-*O*-benzyl-6-deoxy- α -D-glucopyranosyl chloride¹⁵⁷ (**98**) with the protected 2-deoxystreptamine **99**. The 4- (**100**) and 6-glycoside (**101**) were obtained, and from these were prepared **95** and **97**, respectively.

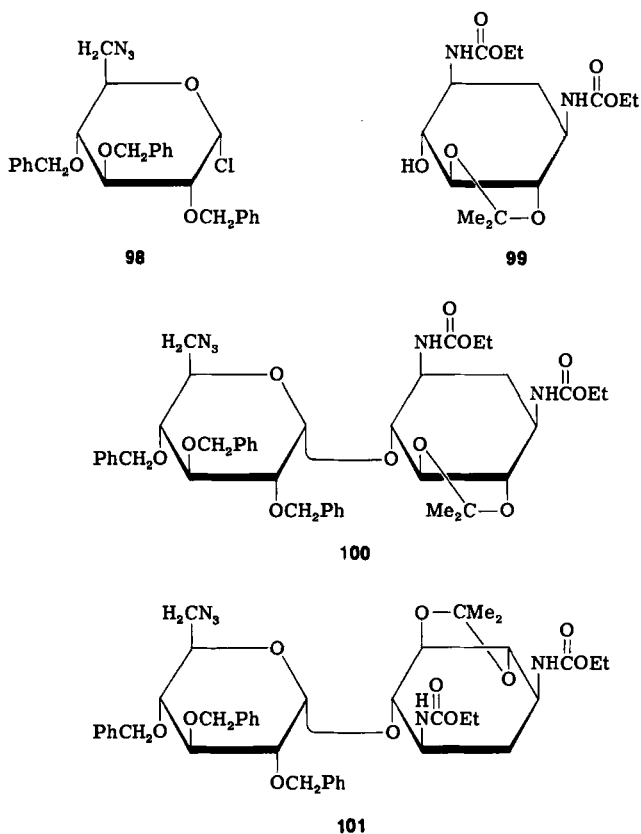
d. Syntheses of Other Glycosyldeoxystreptamines.—6-*O*- α -D-Glucopyranosyl-2-deoxystreptamine (**105**) is a component of the antibiotics NK-1001 and NK-1012-1 (**26** and **27**; see p. 122). This glycoside (**105**) was synthesized by Umezawa and coworkers.¹⁵⁸ Condensation of tetra-*O*-benzyl- α -D-glucopyranosyl chloride¹⁵⁴ with the protected 2-deoxystreptamine **99** afforded, after de-isopropylidenation and acetylation, the 4- (**102**) and 6-glycoside (**104**) in 17 and 30% overall yields; from these were

(155) T. Ueno, N. Kurihara, S. Hashimoto, and M. Nakajima, *Agr. Biol. Chem.* (Tokyo), **31**, 1346 (1967).

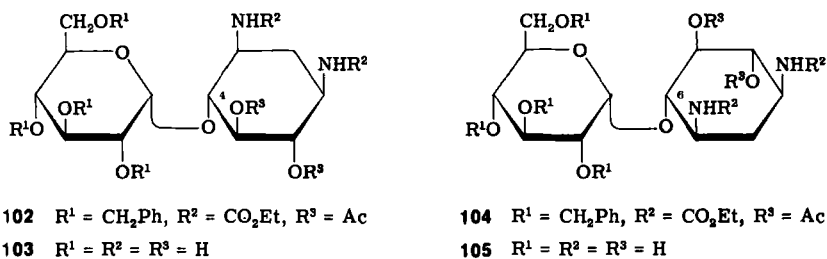
(156) Y. Nishimura, T. Tsuchiya, and S. Umezawa, *Bull. Chem. Soc. Jap.*, **44**, 2521 (1971).

(157) Y. Takagi, T. Tsuchiya, and S. Umezawa, *Bull. Chem. Soc. Jap.*, **46**, 1261 (1973).

(158) Y. Nishimura, T. Tsuchiya, and S. Umezawa, *Bull. Chem. Soc. Jap.*, **43**, 2960 (1970).

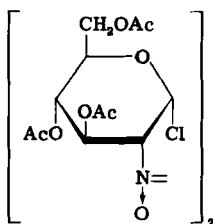


prepared 4-*O*- α -D-glucopyranosyl-2-deoxystreptamine (**103**) and its diastereoisomer (**105**).



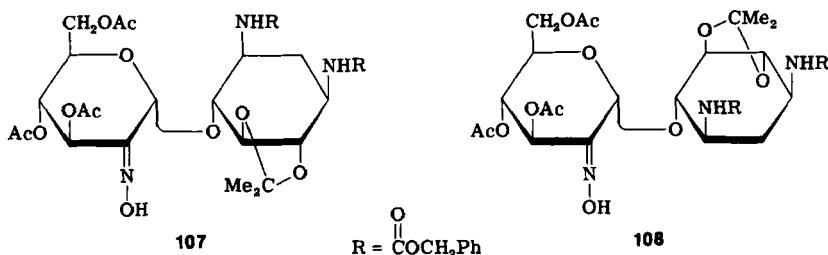
Another synthesis of these two α -D-glycosides was reported by Lemieux and coworkers,¹⁵⁹ who developed a new synthesis of glycosides which involves the reaction^{138,140} of dimeric tri-*O*-acetyl-2-deoxy-2-nitroso- α -D-

glucopyranosyl chloride (106) with alcohols, and this procedure was



106

successfully used to synthesize the deoxystreptamine glycosides. Condensation of 106 with the protected 2-deoxystreptamine 85 in *N,N*-dimethylformamide gave a mixture of the 4- and 6-glycosides (107 and 108) in 53% yield. After simultaneous de-oxygenation and de-isopropyliden-

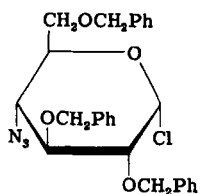


ation with hydrochloric acid, followed by reduction of the resultant ketone group with borohydride, and hydrolysis, the two components were separated by chromatography, affording 103 and 105 in 9 and 17% overall yields, respectively.

By 1973, all of the 4- and 6-*O*-(aminodeoxy- α -D-glucopyranosyl)-2-deoxystreptamines had been synthesized, except for (4-amino-4-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine, and this has now been synthesized by Umezawa and coworkers.¹⁶⁰ 4-Azido-2,3,6-tri-*O*-benzyl-4-deoxy- α -D-glucopyranosyl chloride¹⁵⁷ (109) was condensed with the protected 2-deoxystreptamine 99 to give the 4- (110) and 6-glycoside (111). Subsequent removal of protecting groups gave 4-*O*-(4-amino-4-deoxy- α -D-

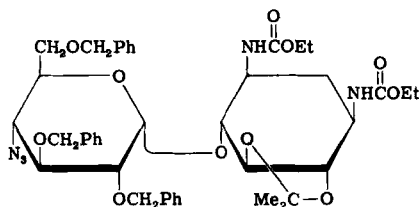
(159) R. U. Lemieux, T. L. Nagabhushan, K. J. Clemetson, and L. C. N. Tucker, *Can. J. Chem.*, **51**, 53 (1973).

(160) Y. Nishimura, T. Tsuchiya, and S. Umezawa, *Bull. Chem. Soc. Jap.*, **46**, 1263 (1973).

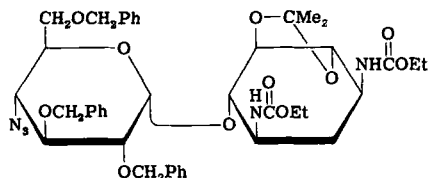


109

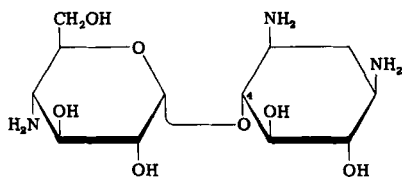
glucopyranosyl)- (112) and 6-O-(4-amino-4-deoxy-D-glucopyranosyl)-2-deoxystreptamine (113), which were devoid of antibacterial activity.



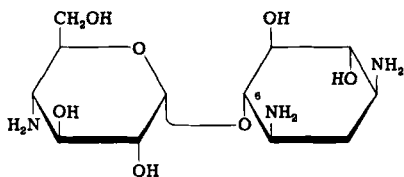
110



111



112

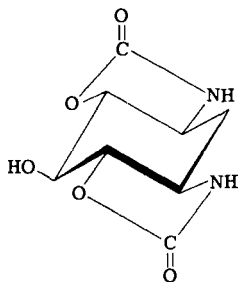


113

5-O-(2-Amino-2-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine (115) was synthesized by Umezawa and Tsuchiya,¹⁶¹ who found that the reaction of (*p*-nitrophenoxycarbonyl or phenoxy carbonyl chloride with 2-deoxystreptamine in the presence of Dowex-1 X-2 (OH⁻) or sodium carbonate in aqueous acetone resulted in the formation of a cyclic carba-

(161) S. Umezawa and T. Tsuchiya, unpublished results.

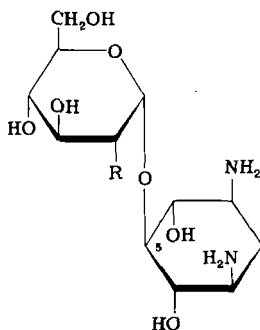
mate (114) in good yield.^{162,163} This reaction was useful for the preparation of 5-*O*-glycosyl-2-deoxystreptamines. Condensation of the cyclic carbamate 114 with 3,4,6-tri-*O*-acetyl-2-deoxy-2-(*p*-methoxybenzylidene-



114

amino)- α -D-glucopyranosyl bromide (see Refs. 174 and 175, p. 153) gave a 5-glycoside, which led to 115. The 5-glycoside 115 had also been obtained by Umezawa and coworkers¹⁶⁴ as a product of the acid reversion of 2-deoxystreptamine with 2-amino-2-deoxy-D-glucose; they found that the 5- α -D-glycoside (115) has significant activity against *Mycobacterium tuberculosis* only (amongst the organisms tested), whereas paromamine (78) shows almost no activity against the same strain.

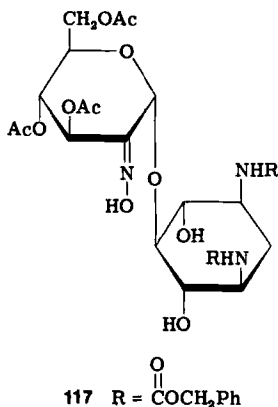
5-*O*- α -D-Glucopyranosyl-2-deoxystreptamine (116) was synthesized

115 R = NH₂

116 R = OH

- (162) S. Umezawa, T. Tsuchiya, and Y. Takagi, *Bull. Chem. Soc. Jap.*, **43**, 1602 (1970).
- (163) S. Umezawa, Y. Takagi, and T. Tsuchiya, *Bull. Chem. Soc. Jap.*, **44**, 1411 (1971).
- (164) S. Umezawa, T. Tsuchiya, and H. Fujita, *J. Antibiot. (Tokyo)*, **A19**, 222 (1966).

by Lemieux and coworkers.¹⁵⁸ Condensation of tri-*O*-acetyl-2-deoxy-2-nitroso- α -D-glucopyranosyl chloride dimer (106, see p. 147) with *N,N'*-di(benzyloxycarbonyl)-2-deoxystreptamine in *N,N*-dimethylformamide gave, as the main product, the oximino compound 117, which, by way of deoxygenation, borohydride reduction, hydrolysis, and hydrogenolysis, afforded 116.



It is noteworthy that the formation of the 5-glycoside 116 is compatible with the formation of 115 by an acid-reversion reaction, and differs from the situation obtaining in the condensation^{165,166} of tetra-*O*-acetyl- α -D-glucopyranosyl bromide [or 2,4,6-tri-*O*-acetyl-3-(benzyloxycarbonyl)amino-3-deoxy- α -D-glucopyranosyl bromide or 2,3,4-tri-*O*-acetyl-6-(benzyloxycarbonyl)amino-6-deoxy- α -D-glucopyranosyl chloride] with *N,N'*-di(benzyloxycarbonyl)-2-deoxystreptamine that yielded 4,6-di-*O*- β -D-glycosides.

e. Synthesis of Kasugamycin.—The total synthesis of kasugamycin was accomplished by H. Umezawa and coworkers^{167,168} and by Nakajima and coworkers^{169,170} in 1968. In the former synthesis, a protected (+)-

(165) S. Umezawa and Y. Ito, *Bull. Chem. Soc. Jap.*, **34**, 1540 (1961).

(166) S. Koto, Y. Ito, and S. Umezawa, *Bull. Chem. Soc. Jap.*, **38**, 1447 (1965).

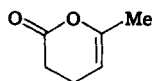
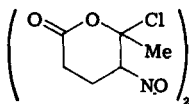
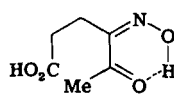
(167) Y. Suhara, F. Sasaki, K. Maeda, H. Umezawa, and M. Ohno, *J. Amer. Chem. Soc.*, **90**, 6559 (1968).

(168) Y. Suhara, F. Sasaki, G. Koyama, K. Maeda, H. Umezawa, and M. Ohno, *J. Amer. Chem. Soc.*, **94**, 6501 (1972).

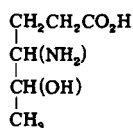
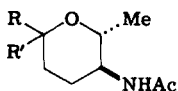
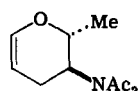
(169) M. Nakajima, H. Shibata, K. Kitahara, S. Takahashi, and A. Hasegawa, *Tetrahedron Lett.*, 2271 (1968).

(170) K. Kitahara, S. Takahashi, H. Shibata, N. Kurihara, and M. Nakajima, *Agr. Biol. Chem. (Tokyo)*, **33**, 748 (1968).

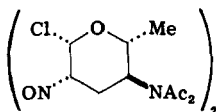
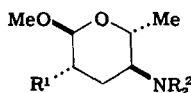
inositol was glycosylated with a 2-nitroso-glycosyl chloride. Addition of nitrosyl chloride to the dihydropyran derivative **118** gave the adduct **119**, which, on hydrolysis, gave the oximino derivative **120**. Catalytic hy-

**118****119****120**

drogenation stereospecifically yielded 4-amino-2,3,4,6-tetradeoxy-DL-*erythro*-hexonic acid (**121**), which, on treatment with acetic anhydride, gave the lactone **122**. Reduction with lithium aluminum hydride gave the hemiacetal **123**, and treatment with acetic anhydride-pyridine gave the dihydropyran derivative **124**. The stereochemistry of this derivative

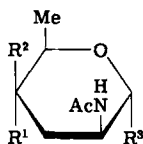
**121****122** R, R' = O**123** R, R' = H, OH**124**

was confirmed by n.m.r. spectral studies, thus verifying that the amino acid **121** was of the *erythro* configuration. Nitrosyl chloride was again added, to give **125**, which, by condensation with methanol, gave the nitroso dimer of **126**; subsequent catalytic hydrogenation, followed by hydrolysis, furnished methyl kasugaminide (**127**). Condensation of **125**

**125****126** R¹ = NO, R² = Ac**127** R¹ = NH₂, R² = H

with 1,2:5,6-di-*O*-isopropylidene-(+)-inositol, followed by catalytic hydrogenation and removal of the protecting groups, gave kasuganobiosamine (**67**). Kasuganobiosamine had previously¹¹³ been converted into kasugamycin. In this elegant synthesis, it is remarkable that not only the displacement, but also the resolution, of **125** was carried out by the protected inositol, yielding a product conforming stereochemically with natural kasuganobiosamine.

In another synthesis of kasuganobiosamine,^{169,170} the *p*-tolylsulfonyloxy group of (the known) methyl 4,6-*O*-benzylidene-3-deoxy-2-*O*-*p*-tolylsulfonyl- α -D-glucopyranoside was converted into an amino group (with inversion) through the intermediate azido derivative, and, after removal of the benzylidene group, the 6-hydroxyl group was replaced by hydrogen by the usual procedure (through the *p*-tolylsulfonyl derivative), yielding the amino sugar **128**. Oxidation with chromic acid gave a 4-ketose, which led, by hydrogenation and the azide-reduction procedure, to methyl *N,N'*-diacetyl- α -kasugaminide (**129**). The glycoside was converted into the glycosyl chloride (**130**), and condensation thereof with the aforementioned, protected inositol afforded kasuganobiosamine.

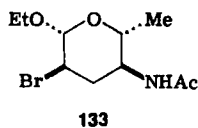
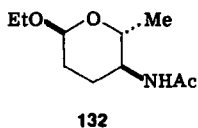
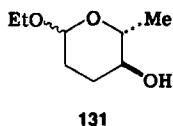


128 $R^1 = H, \quad R^2 = OH, \quad R^3 = OMe$

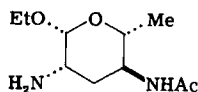
129 $R^1 = NHAc, \quad R^2 = H, \quad R^3 = OMe$

130 $R^1 = NHAc, \quad R^2 = H, \quad R^3 = Cl$

A synthesis of kasugamine has also been reported by Matsumoto and coworkers.¹⁷¹ The (known) 2-ethoxy-3,4-dihydro-6-methyl-2*H*-pyran was hydroborated and the adduct was treated with chloramine to give an amine and a dihydropyranylboric acid. Peroxide oxidation of the latter gave the alcohol **131**, which, by a sequence of reactions involving oxidation to the ketone, formation of the oxime, reduction thereof to the amine, and acetylation, gave the acetamido derivative (**132**). Bromination of **132**, or of the amine obtained by the reaction with chloramine, gave three bromo isomers, two of which were transformed into **133** by treatment with ethanolic hydrogen chloride. Reaction of **133** with sodium azide, and subsequent catalytic hydrogenation, gave the amine **134**. Optical resolution of the amine, followed by acetylation and transglycosylation, gave methyl di-*N*-acetylkasugaminide (**129**).



(171) S. Yasuda, T. Ogasawara, S. Kawabata, and T. Matsumoto, *Tetrahedron Lett.*, 3969 (1969).



134

As a related glycoside, 4-*O*-(6-amino-6-deoxy- β -D-glucopyranosyl)-3-*O*-methyl-(+)-inositol was synthesized; however, it showed no antibiotic activity.¹⁷²

f. Synthesis of α,α -Trehalosamine.—Umezawa and coworkers¹⁷³ synthesized α,α -trehalosamine. Condensation of 3,4,6-tri-*O*-acetyl-2-deoxy-2-(*p*-methoxybenzylidene)amino- α -D-glucopyranosyl bromide^{174,175} with 2,3,4,6-tetra-*O*-benzyl- α -D-glucose,¹⁷⁶ followed by hydrolysis and catalytic hydrogenolysis, yielded α,α -trehalosamine (69, see p. 137).

The analogs 6,6'-diamino-6,6'-dideoxy- α,α -trehalose¹⁷⁷ and 6-amino-6-deoxy- α,α -trehalose¹⁷⁸ were synthesized from trehalose, and found to be devoid of antibacterial activity.

2. Total Synthesis of Aminoglycosides Composed of Three Carbohydrate Moieties

The challenge to synthesize this group of complex, aminoglycoside antibiotics was met by the total synthesis of the kanamycins in 1968; and, later, ribostamycin was synthesized. Also, Umezawa and coworkers synthesized butirosin B and tobramycin, and the total synthesis of dihydrostreptomycin was achieved by Umezawa and coworkers in 1973. Synthesis of related glycosides composed of three carbohydrate moieties will also be described in this Section.

a. Synthesis of Dihydrostreptomycin and Streptomycin.—Following the isolation and determination of the structure of streptomycin some twenty-five years ago, the first total synthesis of the streptomycin series was realized by Umezawa and coworkers¹⁷⁹ in 1974.

(172) M. J. Mohlenkamp and L. Anderson, *J. Org. Chem.*, **33**, 3163 (1968).

(173) S. Umezawa, K. Tatsuta, and R. Muto, *J. Antibiot. (Tokyo)*, **A20**, 388 (1967).

(174) L. Zervas and S. Konstas, *Chem. Ber.*, **93**, 435 (1960).

(175) F. E. Hardy, J. G. Buchanan, and J. Baddiley, *J. Chem. Soc.*, 3360 (1963).

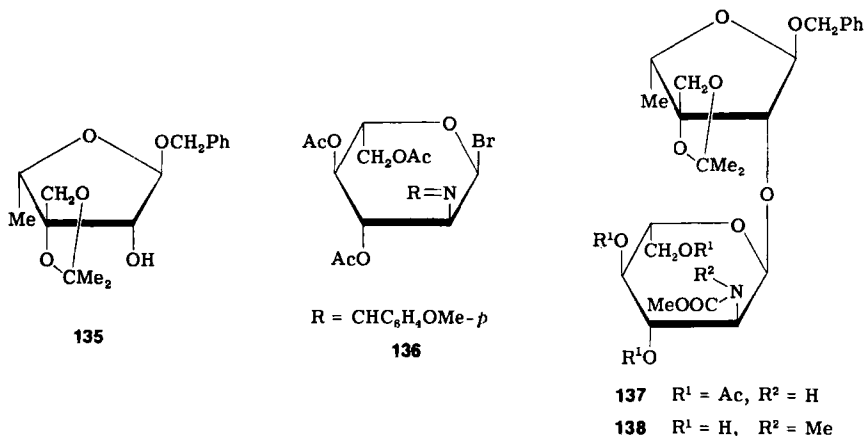
(176) M. E. Tate and C. T. Bishop, *Can. J. Chem.*, **44**, 1801 (1963).

(177) S. Umezawa, T. Tsuchiya, S. Nakada, and K. Tatsuta, *Bull. Chem. Soc. Jap.*, **40**, 395 (1967).

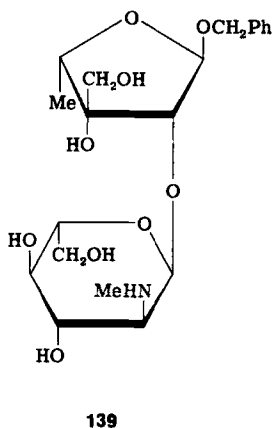
(178) S. Hanessian and P. Lavallée, *J. Antibiot. (Tokyo)*, **25**, 683 (1972).

(179) S. Umezawa, T. Tsuchiya, T. Yamazaki, H. Sano, and Y. Takahashi, *J. Amer. Chem. Soc.*, **96**, 920 (1974).

The isopropylidene derivative (135) of benzyl α -L-dihydrostreptoside and 3,4,6-tri-*O*-acetyl-2-(*p*-methoxybenzylidene)amino-L-glucopyranosyl bromide (136) were prepared from dihydrostreptose^{21,22} and 2-amino-2-deoxy-L-glucose, respectively. Condensation of 135 with 136, followed by cleavage of the Schiff base and by reaction with methyl chloroformate, gave the *N*-(methoxycarbonyl) derivative 137. *N*-Methylation of 137, followed by deacetylation of the product, gave 138. De-



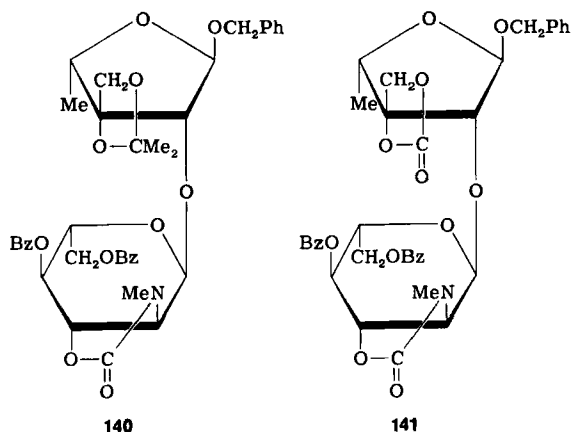
isopropylidenation followed by hydrolysis with barium hydroxide gave benzyl α -L-dihydrostreptobiosaminide (139), identical with a specimen



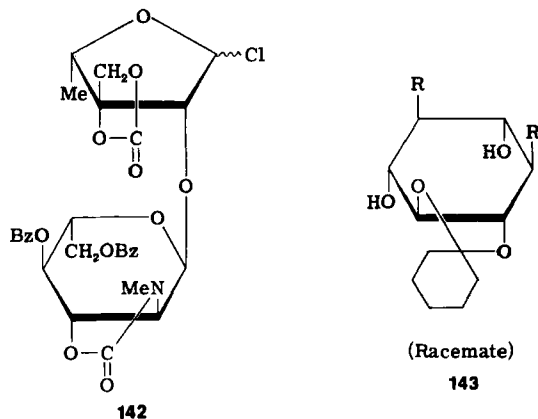
thereof derived¹⁸⁰ from dihydrostreptomycin (2, see p. 115).

(180) G. K. J. Ferguson, I. J. McGilveray, and J. B. Stenlake, *J. Pharm. Pharmacol., Suppl.*, 17, 685 (1965).

The benzyl streptobiosaminide was di-isopropylidenated, and, after the isopropylidene group of the 2-amino-2-deoxy-L-glucose moiety had been selectively hydrolyzed, the two hydroxyl groups liberated were benzoylated. Next, the adjacent methylamino and hydroxyl groups were protected with a cyclic carbamate group¹⁶³ to give **140**. To protect the dihydrostreptose moiety more suitably, the remaining isopropylidene group was now replaced by a carbonate group, affording **141**. Hydro-



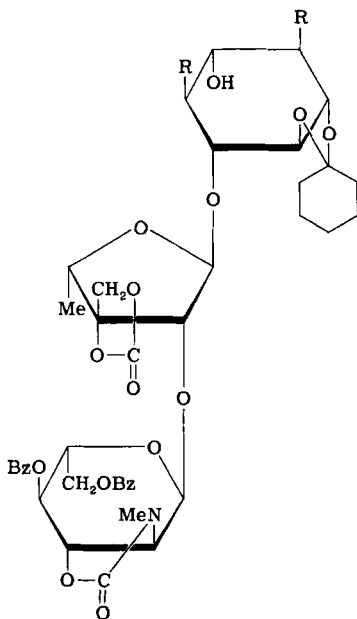
genolysis of the glycoside linkage, followed by reaction with thionyl chloride, furnished the glycosyl chloride (**142**). Also, streptidine¹⁸¹ was transformed into the derivative **143** [R = acetyl(benzoyloxycarbonyl)-



guanidino]. Condensation of **142** with **143** gave **144**, which, after removal

(181) M. L. Wolfrom, S. M. Olin, and W. J. Polglase, *J. Amer. Chem. Soc.*, **72**, 1724 (1950).

of the protecting groups, afforded dihydrostreptomycin^{182,183} (**2**, see p. 115). The synthesis of streptomycin (**1**, see p. 115) was also completed¹⁷⁹ by oxidation of a protected derivative of dihydrostreptomycin with methyl sulfoxide and *N,N'*-dicyclohexylcarbodiimide.

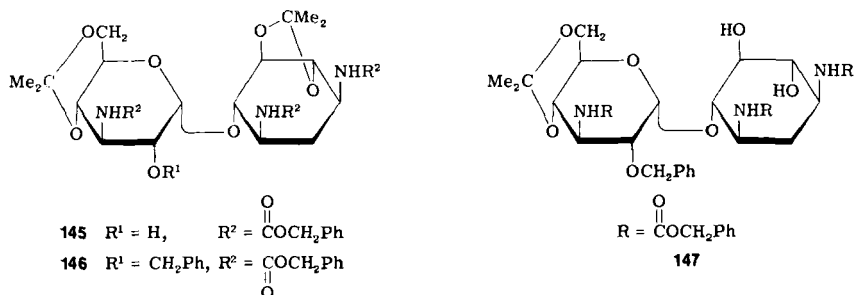


144

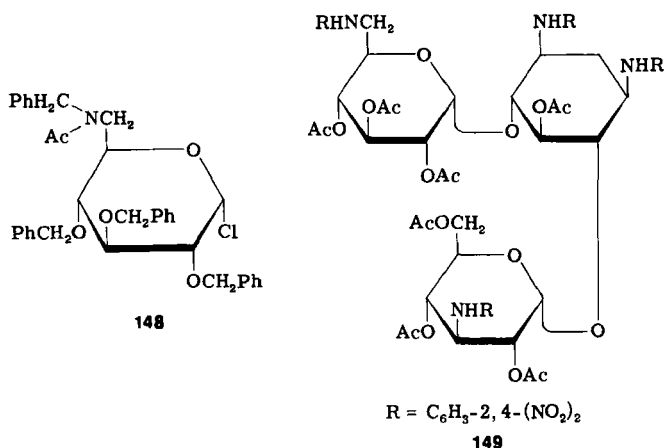
b. Synthesis of Kanamycin A.—Total syntheses of kanamycin A (**23**, see p. 122) have been reported by two groups of workers.^{184–186} Umezawa and coworkers¹⁸⁴ synthesized it by coupling 6-*O*-(3-amino-3-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine¹⁴⁸ (**88**, see p. 144) with 6-amino-6-deoxy-D-glucose by way of their suitably protected derivatives. *N*-Benzyloxycarbonylation of the former, followed by reaction with 2,2-dimethoxypropane, gave the di-isopropylidene derivative **145**, which was

- (182) Q. R. Bartz, J. Controulis, H. M. Crooks, Jr., and M. C. Rebstock, *J. Amer. Soc.*, **68**, 2163 (1946).
- (183) R. L. Peck, C. E. Hoffhine, Jr., and K. Folkers, *J. Amer. Chem. Soc.*, **68**, 1390 (1946).
- (184) S. Umezawa, K. Tatsuta, and S. Koto, *J. Antibiot. (Tokyo)*, **21**, 367 (1968); *Bull. Chem. Soc. Jap.*, **42**, 533 (1969).
- (185) M. Nakajima, A. Hasegawa, N. Kurihara, H. Shibata, T. Ueno, and D. Nishimura, *Tetrahedron Lett.*, 623 (1968).
- (186) A. Hasegawa, N. Kurihara, D. Nishimura, and M. Nakajima, *Agr. Biol. Chem. (Tokyo)*, **32**, 1130 (1968).

benzylated to give 146. The isopropylidene groups were then removed, and partial isopropylidenation with the same reagent at a lower temperature gave the monoisopropylidene derivative (147). Also, methyl



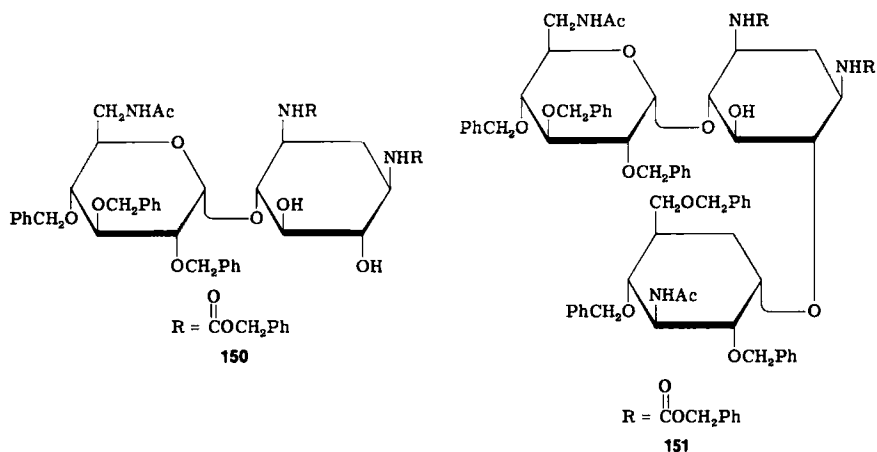
6-acetamido-6-deoxy- α -D-glucopyranoside¹⁸⁷ was benzylated, acetylated, and chlorinated to give the glycosyl halide¹⁵² (148). Condensation of 148 with 147 by a general method,¹⁵⁸ followed by removal of the protecting groups, gave crude kanamycin A. After *N*-(2,4-dinitrophenyl)ation followed by acetylation, chromatography afforded the protected kanamycin A (149), which, after removal of the protecting groups, then gave kanamycin A (23, see p. 122).



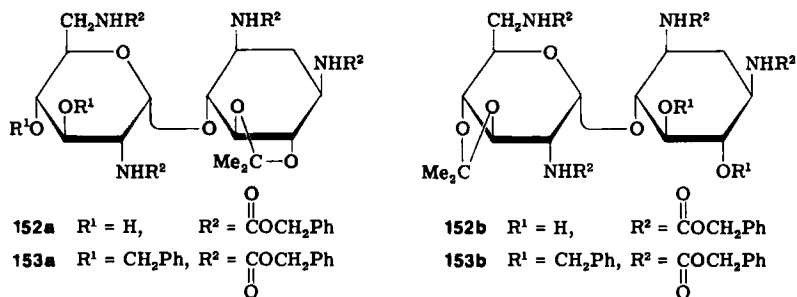
Nakajima and coworkers^{185,186} synthesized kanamycin A by an alternative route. Condensation of 4-*O*-(6-acetamido-2,3,4-tri-*O*-benzyl-6-

(187) F. Cramer, H. Otterbach, and H. Springmann, *Chem. Ber.*, **92**, 384 (1959).

deoxy- α -D-glucopyranosyl)-1,3-di-*N*-(benzyloxycarbonyl)-2-deoxystreptamine¹⁴⁹ (150) with the glycosyl chloride^{149,152} 86 (p. 143) gave protected kanamycin A (151), which led to kanamycin A (23, p. 122).

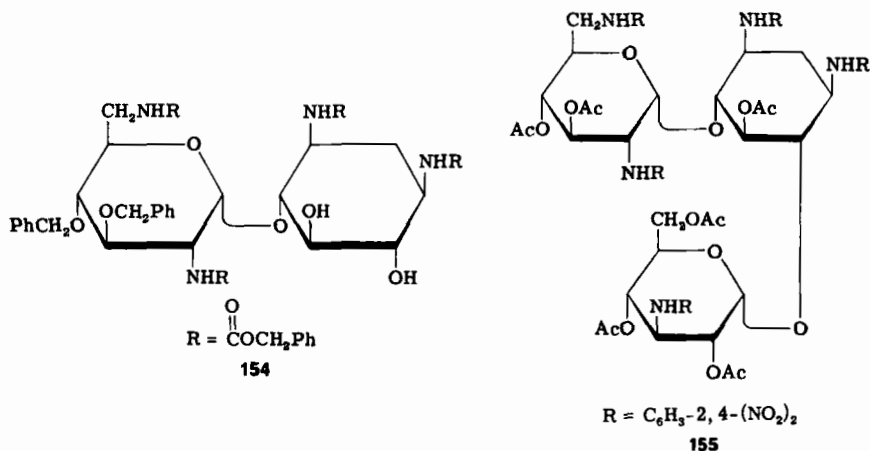


c. Synthesis of Kanamycin B.—Umezawa and coworkers¹⁸⁸ synthesized kanamycin B (24, see p. 122) by coupling neamine¹⁴⁶ (83, see p. 142) with 3-amino-3-deoxy-D-glucose by way of their suitably protected derivatives. Reaction of tetra-*N*-(benzyloxycarbonyl)neamine with one molar proportion of 2,2-dimethoxypropane gave a mixture of two mono-isopropylidene acetals (152a and 152b). Benzylation of the mixture, followed by separation by chromatography, gave the 3',4'-di-*O*-benzyl derivative (153a) and the 5,6-di-*O*-benzyl derivative (153b). De-isopropylidenation of 153a gave 154, which was condensed with the pre-

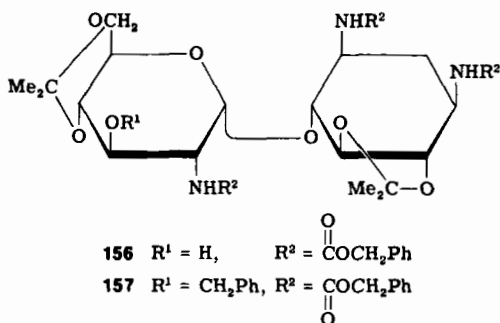


(188) S. Umezawa, S. Koto, K. Tatsuta, H. Hineno, Y. Nishimura, and T. Tsumura, *J. Antibiot. (Tokyo)*, **21**, 424 (1968); *Bull. Chem. Soc. Jap.*, **42**, 537 (1969).

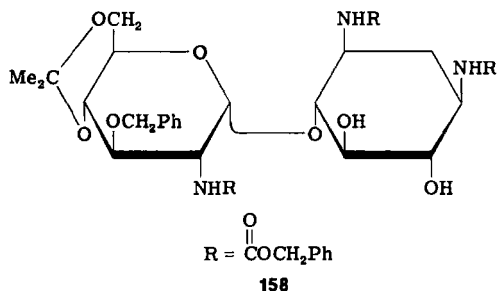
viously mentioned glycosyl chloride¹⁵² **86** (see p. 143). Catalytic hydrogenolysis followed by hydrolysis gave crude kanamycin B. *N*-(2,4-Dinitrophenyl)ation and *O*-acetylation, followed by chromatography, provided a means for securing the pure, protected kanamycin B (**155**), which led to kanamycin B (**24**, p. 122).



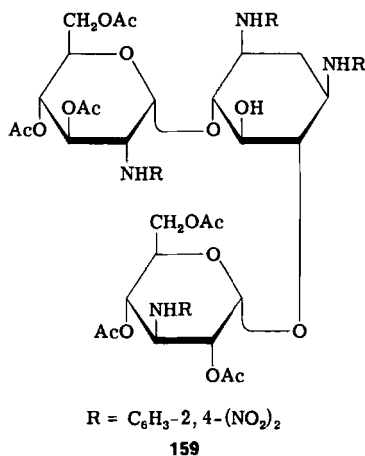
d. **Synthesis of Kanamycin C.**—Umezawa and coworkers¹⁸⁹ synthesized kanamycin C (**25**, see p. 122) from paromamine.^{142,145} Tri-*N*-(benzyloxycarbonyl)paromamine was converted into the di-isopropylidene derivative **156**, which, on benzylation, gave **157**. After de-isopropylidenation of **157**, partial isopropylidenation was performed with 2,2-dimethoxypropane at a lower temperature, to give the monoisopropylidene derivative (**158**). Condensation of **158** with the previously mentioned glycosyl



(189) S. Umezawa, S. Koto, K. Tatsuta, and T. Tsumura, *J. Antibiot. (Tokyo)*, **21**, 162 (1968); *Bull. Chem. Soc. Jap.*, **42**, 529 (1969).

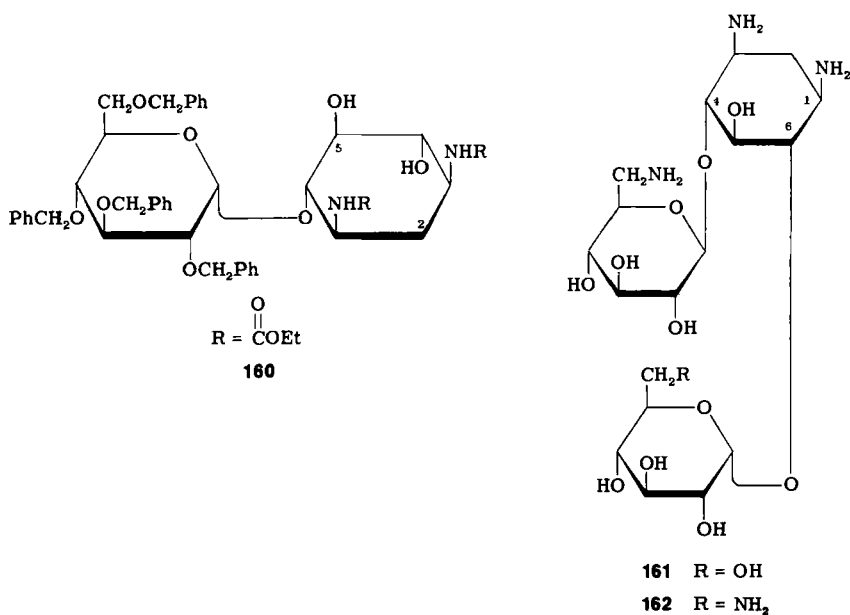


chloride¹⁵² **86** (see p. 143) was followed by de-isopropylidenation with aqueous acetic acid, catalytic hydrogenolysis to remove benzyl and benzyloxycarbonyl groups, and *N*-deacetylation with barium hydroxide. The crude product was (2,4-dinitrophenyl)ated, *O*-acetylated, and subjected to chromatography, to give the protected kanamycin C (**159**), which led to kanamycin C (**25**, p. 122).

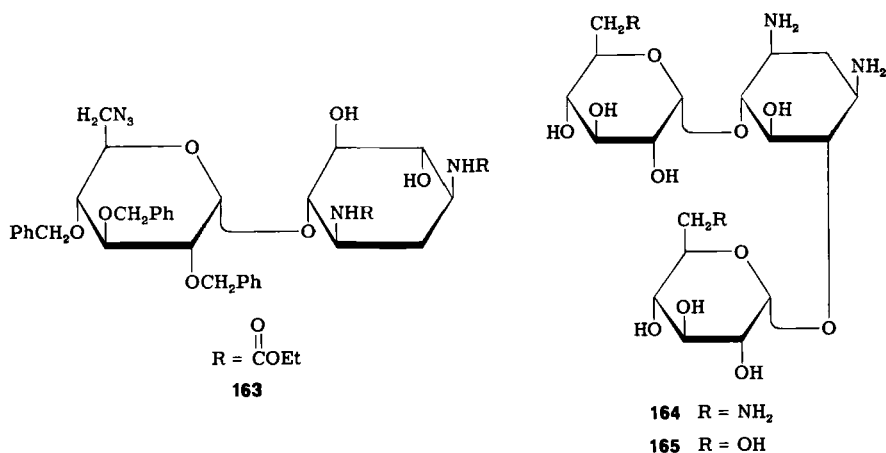


e. Synthesis of Kanamycin-related Glycosides.—The antibiotic, NK-1001 (**26**, see p. 122), was synthesized by Umezawa and coworkers.¹⁵⁶ 1,3-Di-*N*-(ethoxycarbonyl)-6-*O*-(2,3,4,6-tetra-*O*-benzyl- α -D-glucopyranosyl)-2-deoxystreptamine (**160**) was condensed with the previously mentioned glycosyl chloride¹⁵⁷ **98** (see p. 146) to give a 4-*O*- α -D-glycoside and its β -D anomer in 71 and 10% yields, respectively. The former gave, after catalytic hydrogenolysis and removal of the other protecting groups, the antibiotic NK-1001 (**26**). The latter glycoside, on similar treatment, afforded the β -D-glycoside (**161**), which had no antibacterial activity.

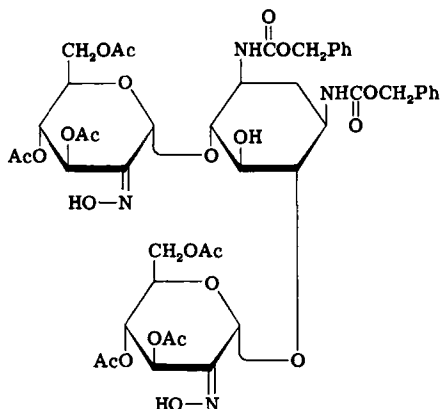
4,6-Di-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine



(164) was synthesized by Umezawa and coworkers.¹⁵⁶ Condensation of the pseudodisaccharide 163 with the previously mentioned glycosyl chloride¹⁵⁷ 98 (see p. 146) gave a 4-*O*- α -D-glycoside and its β anomer in 60 and 19% yields, respectively. Hydrogenolysis, and removal of the other protecting groups from the former, gave 164. The latter similarly gave the β anomer (162), which had no antibacterial activity, whereas 164 had activity.



4,6-Di-*O*- α -D-glucopyranosyl-2-deoxystreptamine (165) was synthesized by Lemieux and coworkers.¹⁵⁹ The diastereoisomeric mixture of the previously mentioned oximino derivatives, 107 and 108, was de-isopropylidenated and the product condensed with the previously mentioned glycosyl chloride (106) to give the pseudotrisaccharide 166, which, after deoximation followed by borohydride reduction, and removal of the protecting groups, gave 165.



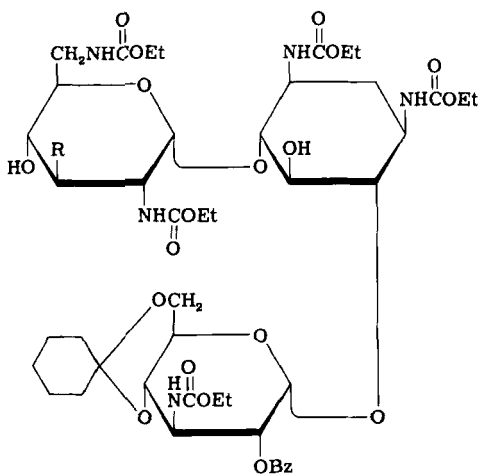
166

f. Synthesis of Tobramycin.—The Umezawas and coworkers¹⁹⁰ synthesized tobramycin from kanamycin B (Ref. 188) (24, see p. 122). The partially protected kanamycin B (190a, see p. 169), gave the mono-*p*-toluenesulfonate 167 having an equatorial *p*-tolylsulfonyloxy group at C-3'. When this compound was treated with sodium iodide in *N,N*-dimethylformamide, the racemic iodo derivative 168 was obtained. Catalytic hydrogenation in the presence of Raney nickel then afforded tobramycin (3'-deoxykanamycin B) (33, see p. 125).

g. Synthesis of Ribostamycin.—Ito and colleagues¹⁹¹ synthesized ribostamycin from neamine^{146,147} (83, see p. 142). The protected neamine (154) was condensed with 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl chloride. In contrast to synthesis of the kanamycin by use of a hexopyranosyl halide, this condensation mainly afforded the 5-glycoside 169, which, after hydrolysis and hydrogenolysis, gave ribostamycin (50, see p. 131).

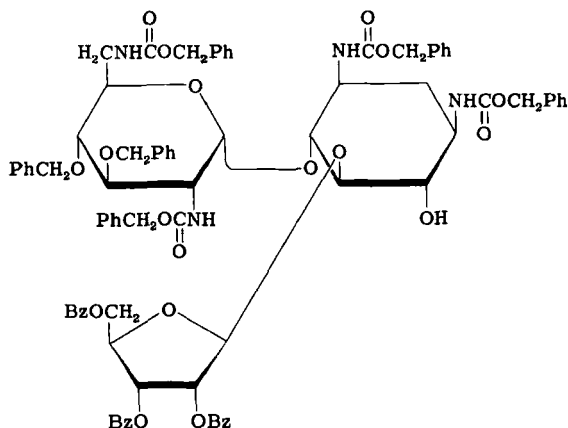
(190) Y. Takagi, T. Miyake, T. Tsuchiya, S. Umezawa, and H. Umezawa, *J. Antibiot.* (Tokyo), **26**, 403 (1973).

(191) T. Ito, E. Akita, T. Tsuruoka, and T. Niida, *Agr. Biol. Chem.* (Tokyo), **34**, 980 (1970); *Antimicrob. Agents Chemother.*, **33** (1970).



167 R = OSO₂C₆H₄Me-*p*

168 R = I (racemic)

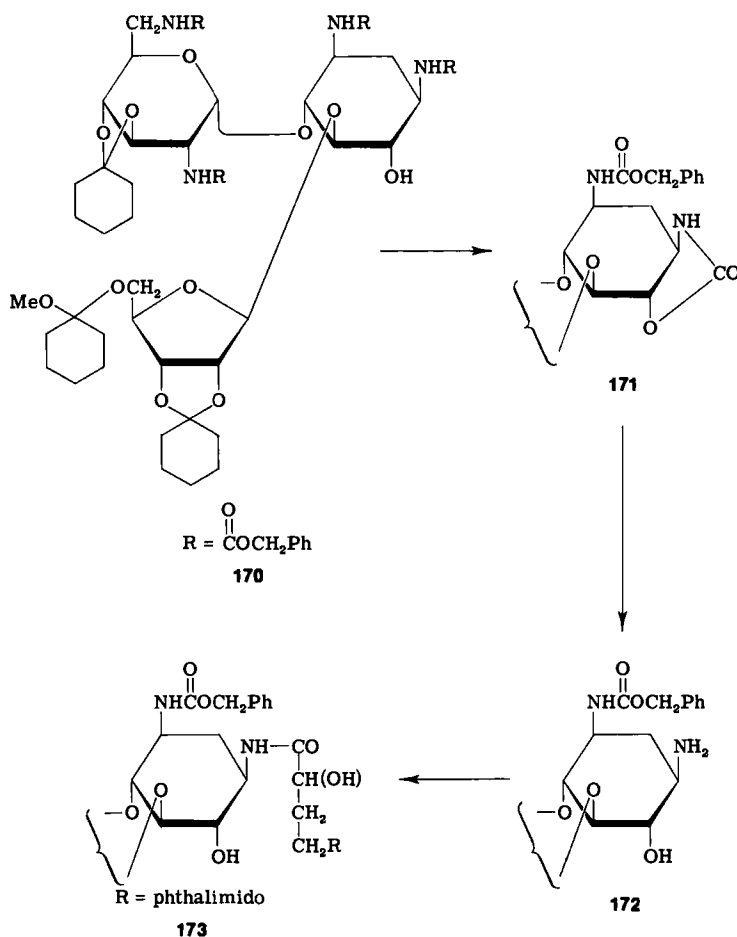


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h. Synthesis of Butirosin B.—The Umezawas and coworkers¹⁸² synthesized butirosin B from ribostamycin.¹⁸¹ Reaction of tetra-*N*-(benzyl-oxy-carbonyl)ribostamycin with cyclohexanone dimethyl acetal gave the tricyclohexylidene derivative **170**, in which one of the cyclohexylidene groups is present in the 1-methoxycyclohexyl form. Treatment of **170** with sodium hydride gave the cyclic carbamate¹⁸³ **171**. Acylation of **172** with 3,4-dideoxy-4-phthalimido-*L*-glycero-tetronic acid [(*S*)-2-hydroxy-

(192) D. Ikeda, T. Tsuchiya, S. Umezawa, and H. Umezawa, *J. Antibiot. (Tokyo)*, **25**, 741 (1972).

4-phthalimidobutyric acid] gave **173**, which on removal of the protecting groups, afforded butirosin B (**56**, see p. 133).



Another synthesis of butirosin B, involving acyl migration, has been reported by Akita and colleagues (see Ref. 218 on p. 174).

IV. CHEMICAL MODIFICATIONS OF AMINOGLYCOSIDE ANTIBIOTICS

1. Synthesis of Aminoglycosides Active against Resistant Bacteria

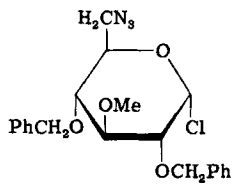
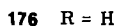
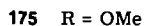
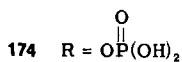
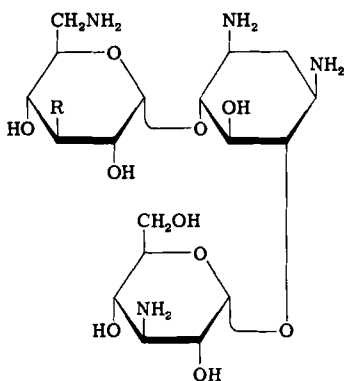
Studies on biochemical mechanisms of resistance to aminoglycoside antibiotics have revealed that they are enzymically inactivated in several

ways, and these mechanisms suggested that chemical modification of certain of the special groups of aminoglycoside molecules might lead to useful derivatives that would be active against resistant bacteria. The new area of these modifications has mostly been developed by Umezawa¹⁹³ and coworkers, who successfully synthesized a series of new antibiotic variants that are remarkably active against resistant bacteria. Moreover, the structures of the butirosins **55** and **56** (see p. 133), discovered by Woo and colleagues,⁹⁶⁻⁹⁸ suggested certain peculiar effects of the presence of the (S)-4-amino-2-hydroxybutyryl residue at N-1 of the 2-deoxystreptamine moiety. These modifications may be divided into (a) deoxy derivatives, (b) 6'-N-methyl and deoxy-6'-N-methyl derivatives, and (c) 1-N-acyl and 1-N-acyl-deoxy derivatives.

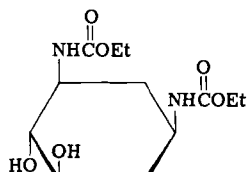
a. Deoxy Derivatives of Aminoglycoside Antibiotics.—A common mechanism of inactivation of kanamycins, neomycins, paromomycins, and ribostamycin by a number of resistant bacteria is phosphorylation of the 3'-hydroxyl group of these antibiotics. In 1967, H. Umezawa and coworkers¹⁹⁴ elucidated the structure (174) of inactivated kanamycin A by enzymic phosphorylation. Similar enzymes have been found not only in Gram-negative bacteria carrying R factor but also in resistant *Pseudomonas* and *Staphylococci*. The phosphorylation mechanism suggested that modification of the 3'-hydroxyl group might lead to derivatives that would be active against resistant bacteria.

There were two approaches to new derivatives, (a) total synthesis or (b) partial synthesis from natural antibiotics. The initial approach by Umezawa and coworkers involved the total synthesis of 3'-O-methylkanamycin A (Ref. 195) (175) and 3'-deoxykanamycin A (Refs. 195a and 196) (176), which involved the condensation of the protected pseudo-disaccharide 179 with the 3-O-methyl- and 3-deoxy-glycosyl chlorides (177 and 178), respectively. The functionalization of the 3'-hydroxyl group caused a remarkable effect on the antibacterial activity. The 3'-deoxykanamycin was as active as the parent antibiotic, and, moreover, it was active against both *Escherichia coli* carrying R factor and resistant *Pseudomonas*, which inactivate kanamycin by the 3'-O-phosphorylation

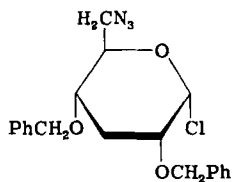
- (193) S. Umezawa, in "XXIIIrd International Congress of Pure and Applied Chemistry," special lectures presented at Boston, Mass., U.S.A., July 26-30, 1971, Butterworth, London, Vol. 2, p. 173.
- (194) H. Umezawa, M. Okanishi, S. Kondo, K. Hamana, R. Utahara, K. Maeda, and S. Mitsuhashi, *Science*, **157**, 1559 (1967).
- (195) (a) S. Umezawa, T. Tsuchiya, R. Muto, and H. Umezawa, *J. Antibiot.* (Tokyo), **24**, 274 (1971). (b) H. Umezawa, T. Tsuchiya, R. Muto, and S. Umezawa, *Bull. Chem. Soc. Jap.*, **45**, 2842 (1972).
- (196) S. Umezawa, Y. Nishimura, H. Hineno, K. Watanabe, S. Koike, T. Tsuchiya, and H. Umezawa, *Bull. Chem. Soc. Jap.*, **45**, 2847 (1972).



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179

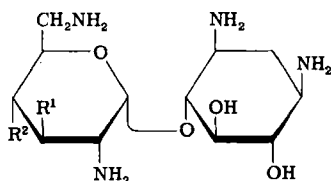


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mechanism, whereas the 3'-*O*-methylkanamycin was almost inactive, suggesting that, although the 3'-hydroxyl group does not play an important role in the mechanism of antibacterial action, there is a strong steric factor associated with the methoxyl group.

3'- and 4'-*O*-Methylneamine (180 and 181) were also prepared,¹⁹⁷ but were found to have markedly decreased activity.

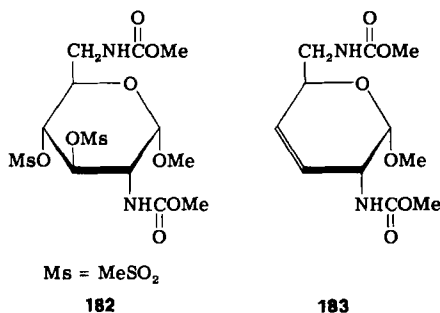
(197) S. Umezawa, T. Jikihara, T. Tsuchiya, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 322 (1972).



180 $R^1 = \text{OMe}, R^2 = \text{OH}$

181 $R^1 = \text{OH}, R^2 = \text{OMe}$

In the second approach, Umezawa and coworkers¹⁹⁸ prepared methyl 2,6-diamino-2,3,4,6-tetradeoxy-D-glucoside (**185**) from methyl 2,6-diamino-2,6-dideoxy-D-glucoside. The Tipson-Cohen procedure,¹⁹⁹ namely, conversion of vicinal disulfonic esters into the corresponding alkenes by treatment with sodium iodide and zinc dust in *N,N*-dimethylformamide, was successfully applied to methyl 2,6-dideoxy-3,4-di-*O*-(methylsulfonyl)-2,6-di(methoxycarbonylamino)- α -D-glucoside (**182**) to give the unsaturated sugar (**183**) in high yield, in accordance with the discovery, by



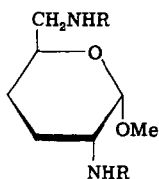
Horton and coworkers,²⁰⁰ that the Tipson-Cohen procedure is applicable to cyclic sugars having *trans*-diequatorial sulfonyloxy groups. Catalytic hydrogenation of the unsaturated sugar gave **184**, and removal of the protecting groups afforded the dideoxyglycoside **185**. This procedure opened the way to the transformation of neamine, kanamycin B, ribostamycin, and the butirosins into their 3',4'-dideoxy derivatives.

Tetra-*N*-(methoxycarbonyl)neamine was selectively cyclohexylidenated and methanesulfonylated to give **186**, and treatment of **186** with sodium iodide and zinc dust, followed by catalytic hydrogenation and removal

(198) S. Umezawa, Y. Okazaki, and T. Tsuchiya, *Bull. Chem. Soc. Jap.*, **45**, 3619 (1972).

(199) R. S. Tipson and A. Cohen, *Carbohydr. Res.*, **1**, 338 (1965).

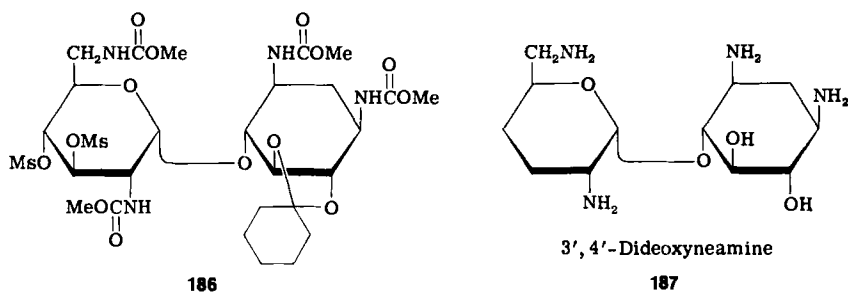
(200) E. Albano, D. Horton, and T. Tsuchiya, *Carbohydr. Res.*, **2**, 349 (1966).



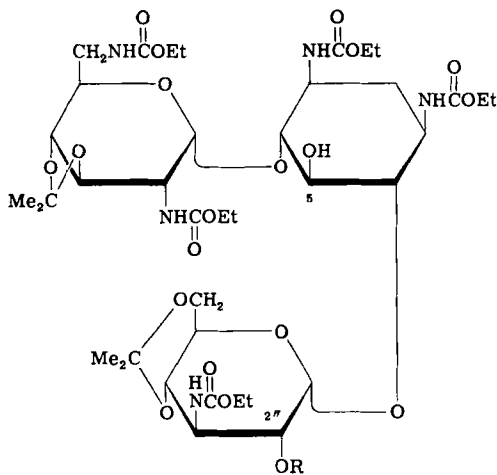
184 R = CO₂Me

185 R = H

of the protecting groups, afforded 3',4'-dideoxyneamine²⁰¹ (**187**), which was found to have activity against resistant bacteria.



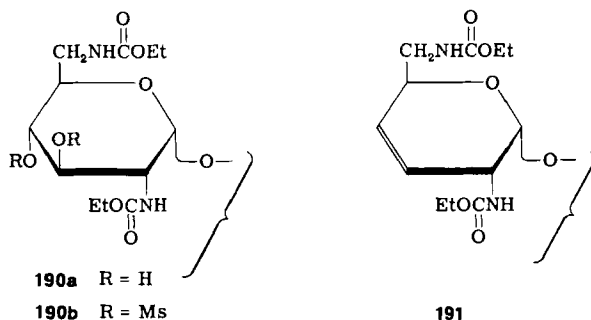
Penta-*N*-(ethoxycarbonyl)kanamycin B was converted into the di-*O*-isopropylidene derivative (**188**). (Alternatively, the hydroxyl groups could be protected with cyclohexylidene groups.) The remaining hydroxyl



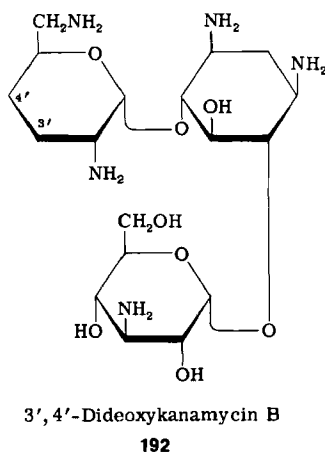
188 R = H

189 R = Bz

group, at C-2'', was benzoylated, to give 189. The sterically hindered hydroxyl group at C-5 remained intact. After removal of the isopropylidene groups, selective isopropylidenation afforded 190a, which, on methanesulfonylation, gave the 3',4'-di-O-(methylsulfonyl) derivative (190b). Application of the Tipson-Cohen procedure¹⁹⁹ then gave the unsaturated derivative 191. Catalytic hydrogenation, followed by removal

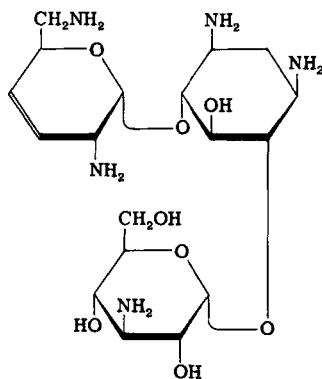


of the protecting groups, furnished^{202,203} 3',4'-dideoxykanamycin B (192), which has significant activity against common bacteria and resistant bacteria, including various strains of *Escherichia coli* carrying R factor and of *Pseudomonas*.



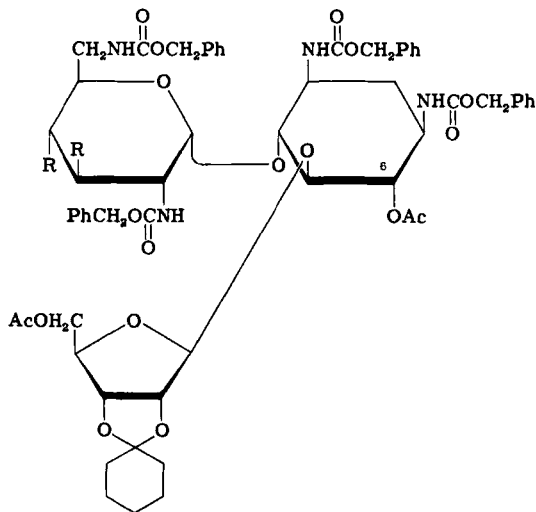
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- (201) S. Umezawa, T. Tsuchiya, T. Jikihara, and H. Umezawa, *J. Antibiot.* (Tokyo), **24**, 711 (1971).
 (202) H. Umezawa, S. Umezawa, T. Tsuchiya, and Y. Okazaki, *J. Antibiot.* (Tokyo), **24**, 485 (1971).
 (203) S. Umezawa, H. Umezawa, Y. Okazaki, and T. Tsuchiya, *Bull. Chem. Soc. Jap.*, **45**, 3624 (1972).

The 3',4'-unsaturated kanamycin B (193) was also prepared from 191, and found to be less active than the parent antibiotic.²⁰⁴



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The remaining 6-hydroxyl group of the tricyclohexylidene derivative (170, see p. 164) of ribostamycin already mentioned was acetylated, and the 1-methoxycyclohexyl group on O-5'' was selectively removed. The 5''-hydroxyl group liberated was then acetylated, and the cyclohexylidene group at O-3',4' was selectively removed, to give 194. Methanesulfonylation of 194 gave 195, and application of the Tipson-Cohen procedure,

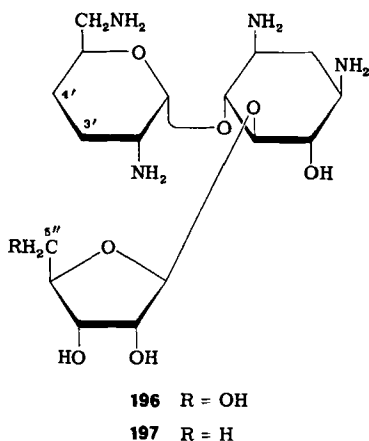


194 R = OH

195 R = OMs

followed by catalytic hydrogenation and removal of the protecting groups, afforded 3',4'-dideoxyribostamycin²⁰⁵ (196), which, as expected, showed activity against various strains of bacteria resistant to ribostamycin.

Because H. Umezawa and coworkers²⁰⁶ had found that an enzyme named phosphate transferase I (which is produced by some strains of resistant *Escherichia coli*) phosphorylates not only the 3'-hydroxyl group of kanamycins but also the 5''-hydroxyl group of ribostamycin and lividomycin A, 5''-deoxy derivatives of ribostamycin and lividomycins were prepared. 3',4',5''-Trideoxyribostamycin (197) was prepared from the



aforementioned compound 170 (see p. 164), and was found to exhibit decreased activity as compared with ribostamycin, indicating that the 5''-hydroxyl group of the D-ribose moiety is important to antibacterial activity.²⁰⁵

The important role of the 5''-hydroxyl group of the D-ribose moiety was also noted with the lividomycins. 5''-Deoxylividomycin A (Ref. 207), B (Ref. 208), and 5''-amino-5''-deoxylividomycin A (Ref. 207) were

(204) S. Umezawa, T. Tsuchiya, and H. Umezawa, unpublished results.

(205) S. Umezawa, T. Tsuchiya, D. Ikeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 613 (1972).

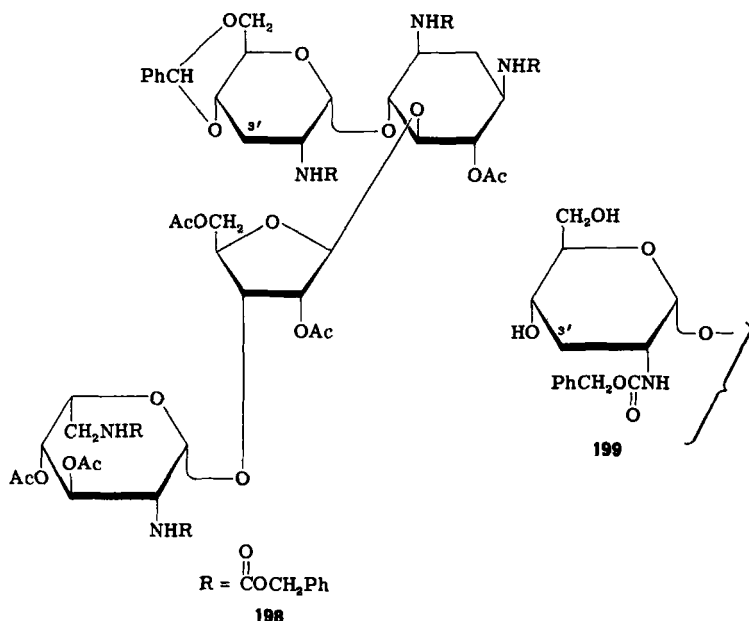
(206) H. Umezawa, H. Yamamoto, M. Yagisawa, S. Kondo, and T. Takeuchi, *J. Antibiot.* (Tokyo), **26**, 407 (1973).

(207) H. Yamamoto, S. Kondo, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 487 (1972).

(208) S. Umezawa, I. Watanabe, T. Tsuchiya, H. Umezawa, and M. Hamada, *J. Antibiot.* (Tokyo), **25**, 617 (1972).

prepared, and found to be much less active than the parent antibiotic.

3'-Deoxyneomycin was prepared²⁰⁹ by selective replacement of the 6'-hydroxyl group of lividomycin B. Benzylidenation of penta-*N*-(benzyl-oxycarbonyl)lividomycin B gave a monobenzylidene derivative, the adjacent hydroxyl groups at C-3 and 4 of the neosamine B moiety not being benzylidenated, suggesting that the neosamine B moiety is present almost in the ¹C₄ or a skew conformation, with the adjacent hydroxyl groups almost axial. Acetylation gave 198, and removal of the benzylidene group from 198 afforded 199. Selective *p*-toluenesulfonylation of the pri-



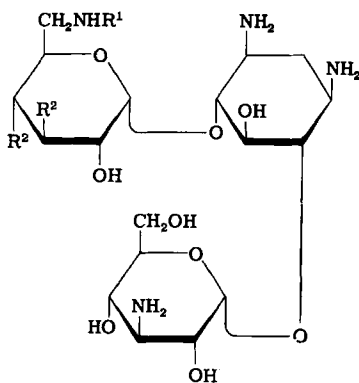
mary hydroxyl group, followed by replacement of the *p*-tolylsulfonyloxy group by an azido group and catalytic hydrogenation of the azide, led to 3'-deoxyneomycin B, which showed, as expected, an antibacterial spectrum broader than that of neomycin, being more active than lividomycin B against various strains of *Pseudomonas aeruginosa* and, expectedly, almost inactive against *Escherichia coli* producing kanamycin phosphate transferase I. Of particular interest is its remarkable activity against *Escherichia coli* JR66/W677, which produces two enzymes, kanamycin phosphate transferase II (Ref. 206) and adenylyl transferase; the former

(209) I. Watanabe, T. Tsuchiya, S. Umezawa, and H. Umezawa, *J. Antibiot.* (Tokyo), **26**, 802 (1973).

phosphorylates the 3'-hydroxyl group, but not the 5''-hydroxyl group of the D-ribose moiety (as it differs from kanamycin phosphate transferase I), and the latter adenylylates the 2''-hydroxyl group of the kanamycins and gentamicins.²¹⁰

2''-Deoxygentamicin C₂ was synthesized by Daniels²¹¹ and found, as expected, to be active against resistant bacteria producing the adenylyl transferase.

b. 6'-N-Methyl and Deoxy-6'-N-methyl Derivatives of Kanamycin B.—A second form of enzymic inactivation of aminoglycoside antibiotics is N-acetylation. As reported by H. Umezawa and coworkers,²¹² the inactivated product of kanamycin A is its 6'-N-acetyl derivative (**200**). Therefore, modification of the 6'-amino group was studied. Selective 6'-N-(benzyloxycarbonyl)ation was achieved by use of benzyl *p*-nitrophenyl carbonate, to give **201** in a yield of 60%. The benzyloxycarbonyl group was reduced to a methyl group by lithium aluminum hydride, to afford 6'-N-methylkanamycin A (Ref. 213) (**202**). 3',4'-Dideoxykanamycin B (**192**) was also converted into its 6'-N-methyl derivative²¹³ (**203**). The 6'-N-methyl derivatives showed activity at the same levels as those of the parent substances, and, as expected, were active against resistant bacteria producing the acetyl transferase. Moreover, the 3',4'-dideoxy-6'-N-methylkanamycin B (**203**) showed remarkable activity



200 R¹ = Ac, R² = OH

201 R¹ = COOCH₂Ph,
R² = OH

202 R¹ = Me, R² = OH

203 R¹ = Me, R² = H

(210) R. Benveniste and J. Davies, *FEBS Lett.*, **14**, 293 (1971).

(211) P. J. L. Daniels, J. Weinstein, R. W. Tkach, and J. Morton, *J. Antibiot.* (Tokyo), **27**, 150 (1974).

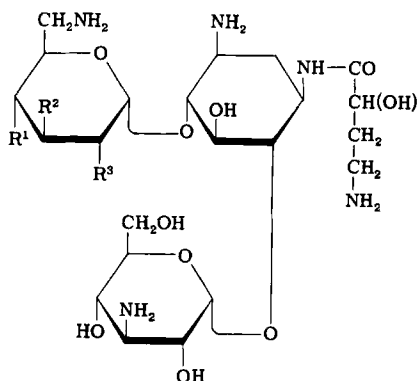
against a *Pseudomonas* which inactivate kanamycins by the 3'-O-phosphorylation mechanism, showing that the combination of 3',4'-deoxygenation and 6'-N-methylation may provide activity against many resistant bacteria.

c. 1-N-Acyl and 1-N-Acyl-deoxy Derivatives of Aminoglycoside Antibiotics.—Another modification of aminoglycoside antibiotics was suggested by the presence of the (S)-4-amino-2-hydroxybutyryl side chain in the butirosins. Butirosins (**55** and **56**, see p. 133) have activity spectra broader than that of ribostamycin (**50**, see p. 131), as they inhibit some kanamycin-resistant bacteria, and it has been confirmed that acylation of the amino group on C-1 with this peculiar amino acid inhibits kanamycin phosphate transferase I; however, kanamycin phosphate transferase II phosphorylates the hydroxyl group of the butirosins.

Modification of kanamycin A in this way was reported by Kawaguchi and coworkers,²¹⁴ and the kanamycin derivative named BB-K8 (**204**) was found to be active against both kanamycin-sensitive and -resistant organisms, inhibiting both kanamycin phosphate transferases I and II. Selective 6'-N-(benzyloxycarbonyl)ation of kanamycin A with N-(benzyloxycarbonyloxy)succinimide, followed by N-acylation with an active ester of L(-)-4-(benzyloxycarbonyl)amino-2-hydroxybutyric acid and separation by chromatography afforded BB-K8 (**204**). Configurational and positional isomers of BB-K8 were also prepared.²¹⁵ The analogs of kanamycin B (Ref. 216) (**205**), 3',4'-dideoxykanamycin B (Ref. 216) (**206**), tobramycin²¹⁷ (**207**), paromomycin I (Ref. 217), and neamine^{218,219} (**208**) also showed improved activity against resistant organisms.

Haskell and coworkers²²⁰ modified butirosin A (**55**, see p. 133) in its

-
- (212) H. Umezawa, M. Okanishi, R. Utahara, K. Maeda, and S. Kondo, *J. Antibiot.* (Tokyo), **A20**, 136 (1967).
(213) H. Umezawa, Y. Nishimura, T. Tsuchiya, and S. Umezawa, *J. Antibiot.* (Tokyo), **25**, 743 (1972).
(214) H. Kawaguchi, T. Naito, S. Nakagawa, and K. Fujisawa, *J. Antibiot.* (Tokyo), **25**, 695 (1972).
(215) T. Naito, S. Nakagawa, Y. Abe, S. Toda, K. Fujisawa, T. Miyaki, H. Koshiyama, H. Ohkuma, and H. Kawaguchi, *J. Antibiot.* (Tokyo), **26**, 297 (1973).
(216) S. Kondo, K. Iinuma, H. Yamamoto, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **26**, 412 (1973).
(217) I. R. Hooper, *Abstr. Papers Amer. Chem. Soc. Meeting*, **165**, MEDI 7 (1973).
(218) E. Akita, Y. Horiuchi, and S. Yasuda, *J. Antibiot.* (Tokyo) **26**, 365 (1973).
(219) H. Tsukiura, K. Fujisawa, M. Konishi, K. Saito, K. Numata, H. Ishikawa, T. Miyaki, K. Tomita, and H. Kawaguchi, *J. Antibiot.* (Tokyo), **26**, 351 (1973).
(220) T. H. Haskell, R. Rodebaugh, N. Plessas, D. Watson, and R. D. Westland, *Carbohydr. Res.*, **28**, 263 (1973).

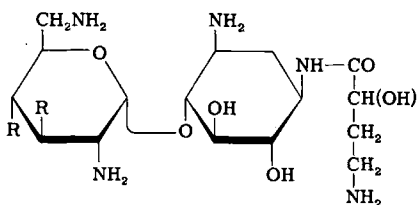


204 (BB-K8), $R^1 = R^2 = R^3 = \text{OH}$

205 $R^1 = R^2 = \text{OH}$, $R^3 = \text{NH}_2$

206 $R^1 = R^2 = \text{H}$, $R^3 = \text{NH}_2$

207 $R^1 = \text{OH}$, $R^2 = \text{H}$, $R^3 = \text{NH}_2$



208 $R = \text{OH}$

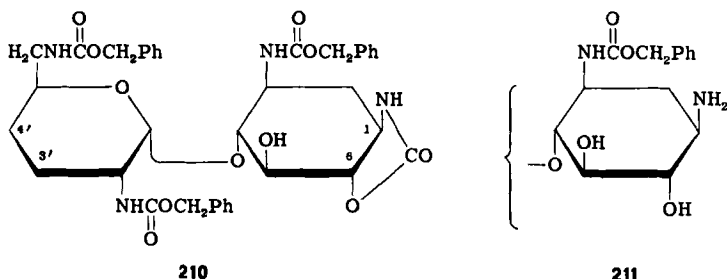
209 $R = \text{H}$

aminoacyl side-chain. Each of the four amino groups was blocked with a 5,5-dimethyl-3-oxo-1-cyclohexen-1-yl group, and, after alkaline cleavage of the amido linkage, the amino group on C-1 was reacylated with a variety of amino acids.

The selective acylation of the amino group on C-1 of the 2-deoxystreptamine moiety is, however, generally difficult; for 3',4'-dideoxyneamine (187), ribostamycin (50, see p. 131), and lividomycin A (18, see p. 120), it has been achieved through a cyclic carbamate intermediate.

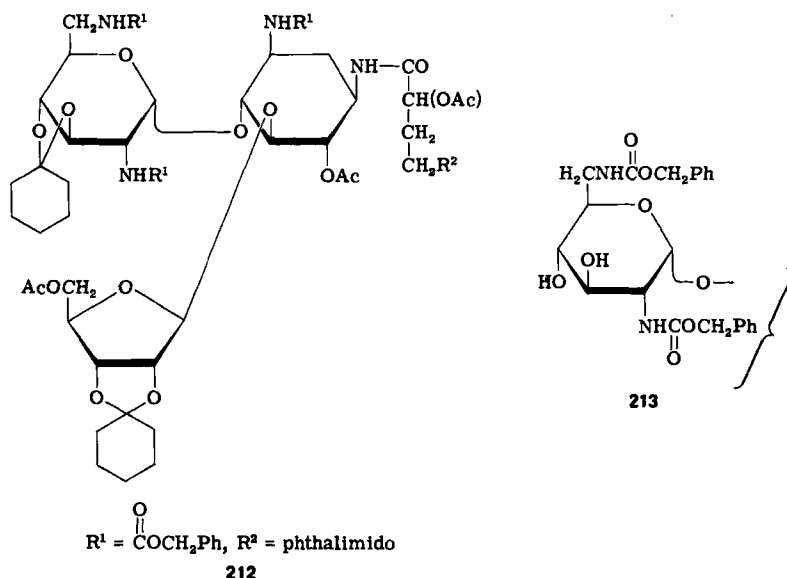
When tetra-*N*-(benzyloxycarbonyl)-3',4'-dideoxyneamine was treated with sodium hydride in *N,N*-dimethylformamide under nitrogen, the 1,6-carbamate (210) was obtained.²²¹ Selective hydrolysis of the carbamate gave 211, and acylation of the amino group on C-1, followed by removal

(221) S. Umezawa, D. Ikeda, T. Tsuchiya, and H. Umezawa, *J. Antibiot.* (Tokyo), **26**, 304 (1973).



of the protecting groups, afforded 1-*N*-[(*S*)-4-amino-2-hydroxybutyryl]-3',4'-dideoxyneamine (209), which showed improved activity against resistant bacteria.

Butirosin B was synthesized from ribostamycin (see Section III,2,*h*, p. 163). 3',4'-Dideoxybutirosin B was synthesized²²² from the already mentioned derivative 173 (see p. 164) of ribostamycin. Selective hydrolysis of the 5"-*O*-(1-methoxycyclohexyl) linkage followed by acetylation gave the tri-*O*-acetyl derivative (212), which, by selective removal of the cyclohexylidene group at *O*-3',4', afforded 213. Methane-

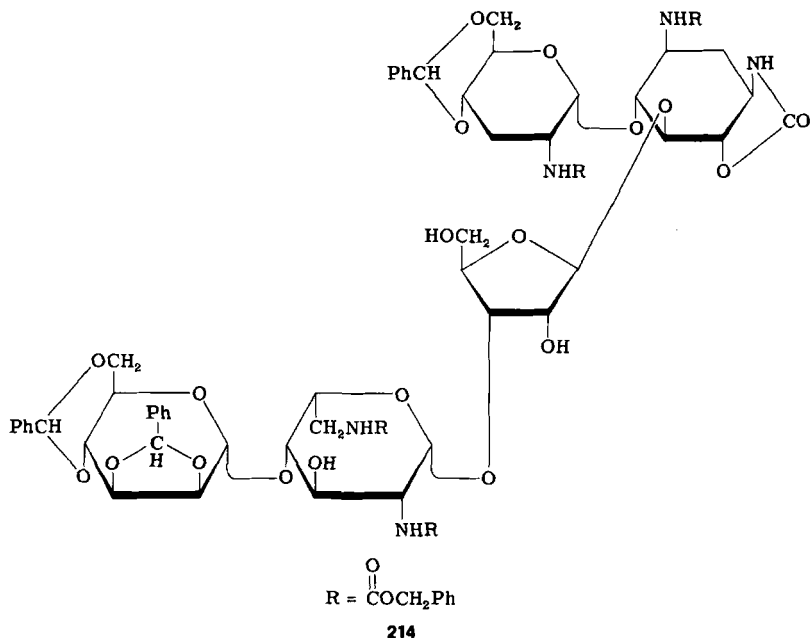


sulfonylation of the 3'- and 4'-hydroxyl groups and application of the

(222) D. Ikeda, T. Tsuchiya, S. Umezawa, H. Umezawa, and M. Hamada, *J. Antibiot.* (Tokyo), **26**, 307 (1973).

Tipson-Cohen procedure¹⁹⁸⁻²⁰⁰ to the product led to the formation of 3',4'-dideoxybutirosin B, which showed enhanced activity as compared with ribostamycin and 3',4'-dideoxyribostamycin, and which was, moreover, effective against bacteria that inactivate the butirosins by phosphorylation of the 3'-hydroxyl group, indicating that the combination of 3',4'-deoxygenation and 1-*N*-acylation provides broad activity against resistant organisms.

Lividomycin A is pentacyclic and is structurally more complex than lividomycin B; however, the former was suitable for 1-*N*-acylation. Reaction of penta-*N*-(benzyloxycarbonyl)lividomycin A with benzaldehyde dimethyl acetal gave a tribenzylidene derivative which, on treatment with sodium hydride in *N,N*-dimethylformamide, gave the cyclic carbamate **214**. Of importance was the fact that, although there are two pairs



of adjacent hydroxyl and (benzyloxycarbonyl)amino groups in the tribenzylidene derivative, one pair, that in the neosamine B moiety, remained intact in this reaction, suggesting that the neosamine B may exist in the ¹C₄ or a skew conformation, with the adjacent groups axial. Selective hydrolysis of the carbamate, followed by 1-*N*-acylation, led²²³

(223) I. Watanabe, T. Tsuchiya, S. Umezawa, and H. Umezawa, *J. Antibiot.* (Tokyo), **26**, 310 (1973).

to the formation of 1-*N*-[(*S*)-4-amino-2-hydroxybutyryl]lividomycin A, which has, as expected, a broader spectrum of activity than the parent antibiotic, as it inhibits resistant bacteria producing kanamycin phosphate transferase I or II, or adenylyl transferase.

2. Other Chemical Modifications

Derivatives of aminoglycoside antibiotics prepared by partial syntheses other than those mentioned in the preceding Section will be reviewed in this Section.

a. Streptomycin Derivatives.—(1) A hydrogenation product, methylstreptomycin²²⁴ (7, see p. 115) in which the aldehyde group has been replaced by a methyl group, (2) an addition product of streptomycin with nitromethane in which the aldehyde group was converted into $\text{—CH(OH)—CH}_2\text{NO}_2$, (3) its reduction product having a $\text{CH(OH)—CH}_2\text{NH}_2$ group, and (4) its oxidation product (prepared with nitric acid) in which the side-chain was deaminated to $\text{CH(OH)—CH}_2\text{OH}$ along with nitrosation of the methylamino group to —N(NO)Me , were reported by Heding and coworkers.²²⁵ These derivatives were found to be less active than dihydrostreptomycin, and a nitrosation product of dihydrostreptomycin in which the methylamino group was changed to —N(NO)Me had no antibacterial activity. Reduction of streptomycin with aluminum amalgam to yield dihydrodeoxystreptomycin²²⁶ (5, see p. 115) has been studied in detail.^{227,228}

Several glycosides were prepared¹⁸⁰ from methyl streptobiosaminide. Dideguanylstreptomycylamine²²⁹ was prepared by alkaline degradation of streptomycylamine (obtained by reduction of streptomycin oxime).

Weakly active *N,N'*-(dialkoxyphenylthio)carbamoyle-dideamidino-dihydrostreptomycin,²³⁰ long-acting 1,3-dibenzyl-2-streptomycin-imidazolidine,²³¹ and the antiviral undecabenzoylstreptomycin²³² were reported.

(224) H. Heding, *Tetrahedron Lett.*, 2831 (1969).

(225) H. Heding, G. J. Fredericks, and O. Lützen, *Acta Chem. Scand.*, **26**, 3251 (1972).

(226) H. Ikeda, *Proc. Japan Acad.*, **32**, 48, 53 (1956).

(227) I. Fujimaki and K. Tsuji, *Nippon Noget Kagaku Kaishi*, **45**, 137 (1971); *Chem. Abstr.*, **75**, 88,867 (1971); and earlier papers.

(228) K. Tsuji and I. Fujimaki, *Tetrahedron Lett.*, 4229 (1970).

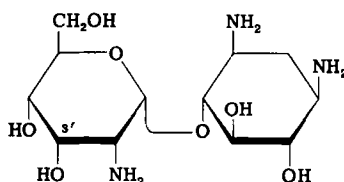
(229) P. Claes, H. Vanderhaeghe, and L. Verlooy, *Bull. Soc. Chim. Belges*, **80**, 659 (1971).

(230) E. N. Lazareva, V. N. Golubev, A. N. Shneerson, and O. S. Vasilenko, *Antibiotiki*, **13**, 682 (1968).

(231) V. N. Golubev, V. G. Koroleva, V. K. Vasiliev, and E. N. Lazareva, *Antibiotiki*, **15**, 491 (1970).

b. Derivatives of Neomycin B, Paromomycin I, and Paromamine.—A series of mono-*N*-alkyl and aralkyl derivatives of neomycin B and paromomycin I were prepared by borohydride reduction of the corresponding Schiff bases.²³³

In view of the important role of the 3'-hydroxyl group in the mechanism of inactivation by resistant bacteria (see p. 165), Hanessian and co-workers²³⁴ prepared 3'-epiparomamine. The already mentioned derivative of paromamine (156, see p. 159) was oxidized with methyl sulfoxide-acetic anhydride to give a glycos-3'-ulose derivative, which, by borohydride reduction and removal of the protecting groups, led to 215.



3'-Epiparomamine

215

c. Derivatives of Kanamycin A.—H. Umezawa and coworkers²³⁵ prepared a series of tetra-*N*-alkyl and aralkyl derivatives of kanamycin A (as lipophilic derivatives) by borohydride reduction of the corresponding Schiff bases. Among the derivatives, tetra-*N*-(*p*-chlorobenzyl)kanamycin A was found to have marked activity against *Pseudomonas*. *N*-Methyl derivatives of kanamycin A were also reported by Claes and Vanderhaeghe.²³⁶

N-Methanesulfonate derivatives of kanamycin A and neomycin have been prepared,^{237,238} and studied biologically in detail.²³⁹⁻²⁴¹

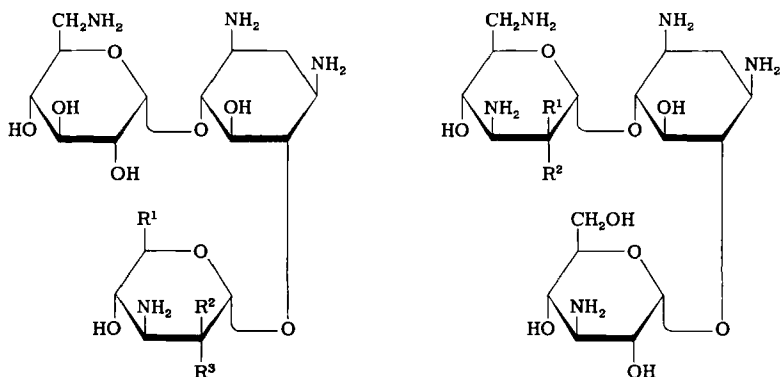
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- (232) E. N. Lazareva, V. N. Golubev, K. I. Germanova, T. Ya. Goncharskaya, O. S. Vasilenko, and P. S. Braginskaya, *Antibiotiki*, **15**, 727 (1970).
 - (233) L. Penasse, P. Barthelemy, and C. Nominé, *Bull. Soc. Chim. Fr.*, 2391 (1969).
 - (234) S. Hanessian, R. F. Butterworth, and T. Nakagawa, *Carbohydr. Res.*, **26**, 261 (1973).
 - (235) A. Fujii, K. Maeda, and H. Umezawa, *J. Antibiot. (Tokyo)*, **21**, 340 (1968).
 - (236) P. Claes and H. Vanderhaeghe, *Bull. Soc. Chim. Belges*, **78**, 561 (1969).
 - (237) S. Umezawa, Y. Ito, S. Fukatsu, and H. Umezawa, *J. Antibiot. (Tokyo)*, **A12**, 114 (1959).
 - (238) T. Tsuchiya and S. Umezawa, *J. Antibiot. (Tokyo)*, **A15**, 73 (1962).
 - (239) H. Umezawa, T. Takeuchi, S. Yamazaki, K. Nitta, T. Osawa, and S. Umezawa, *J. Antibiot. (Tokyo)*, **A12**, 117 (1959).
 - (240) M. H. Pindell and J. Lein, *Chemotherapy*, **8**, 163 (1964).
 - (241) J. R. Boissier, J. Philippe, P. Destombes, O. Dumont, Y. Boilot, M. M. Braun, and C. Carfantan, *Toxicol. Appl. Pharmacol.*, **7**, 190 (1965).

Modification of the primary, 6''-hydroxyl group of kanamycin A has been extensively studied by Umezawa and coworkers. Tetra-*N*-(benzyl-oxy-carbonyl)kanamycin A was selectively *p*-toluenesulfonylated or naphthalenesulfonylated at O-6'' and the product converted into the corresponding chloro (216) and iodo derivative.²⁴² Catalytic hydrogenation of the iodo derivative gave²⁴³ 6''-deoxykanamycin A (217). These 6''-halogeno and 6''-deoxy derivatives have an activity similar to that of the parent antibiotic. Kanamycin-6''-uronic acid (218), its esters, and amide,²⁴⁴ and kanamycin 6''-phosphate (Ref. 245) (219) and 4'',6''-*O*-benzylidene-kanamycin²⁴⁶ were prepared and found to have decreased antibacterial activity.

6''-Amino-6''-deoxy- and 6''-deoxy-6''-hydrazinokanamycin A (220 and 221) were prepared²⁴⁷ and found to have activity. Inouye^{248,249} prepared some derivatives in which one of the amino sugar moieties had been changed to the *D-manno* configuration. Partial *p*-toluenesulfonylation of tetra-*N*-acetylkanamycin A gave the 2'',6''-di-*O-p*-tolylsulfonyl derivative, which, through a monoazido derivative, afforded 222. Analogous reaction with hydrazine gave 223. Nitromethane cyclization of a dialdehyde obtained by periodate oxidation of tetra-*N*-acetylkanamycin A gave a mixture of 3'-deoxy-3'-nitro derivatives, from which, after catalytic hydrogenation followed by chromatography and hydrazinolysis, 3'-amino-3'-deoxy-2'-*L*-glycero-kanamycin A (224) and 3'-amino-3'-deoxykanamycin A (225) were obtained.²⁴⁹

d. Gentamicin Derivatives.—A series of Schiff bases of gentamicin C₂ was prepared.²⁵⁰ The adjacent hydroxyl and methylamino groups formed an oxazolidine, five different aldehydes being incorporated into each of the various Schiff bases. Borohydride reduction of the benzaldehyde Schiff base led to tetra-*N*-benzylgentamicin C₂, which has no antibacterial activity.

- (242) T. Tsuchiya and S. Umezawa, *Bull. Chem. Soc. Jap.*, **38**, 1181 (1965).
- (243) T. Tsuchiya, S. Iriyama, and S. Umezawa, *J. Antibiot. (Tokyo)*, **A16**, 173 (1963).
- (244) T. Kobayashi, T. Tsuchiya, K. Tatsuta, and S. Umezawa, *J. Antibiot. (Tokyo)*, **23**, 225 (1970).
- (245) S. Umezawa, K. Tatsuta, T. Tsuchiya, and E. Yamamoto, *Bull. Chem. Soc. Jap.*, **40**, 1972 (1967).
- (246) K. Tatsuta, K. Kobayashi, and S. Umezawa, *J. Antibiot. (Tokyo)*, **A20**, 267 (1967).
- (247) S. Inouye, *J. Antibiot. (Tokyo)*, **A20**, 6 (1967).
- (248) S. Inouye, *Chem. Pharm. Bull. (Tokyo)*, **15**, 1888 (1967).
- (249) S. Inouye, *Chem. Pharm. Bull. (Tokyo)*, **16**, 573 (1968).
- (250) D. J. Cooper, J. Weinstein, and J. A. Waitz, *J. Med. Chem.*, **14**, 1118 (1971).



216 R¹ = CH₂Cl, R² = H, R³ = OH

217 R¹ = Me, R² = H, R³ = OH

218 R¹ = CO₂H, R² = H, R³ = OH

219 R¹ = CH₂OPO(OH)₂, R² = H, R³ = OH

220 R¹ = CH₂NH₂, R² = H, R³ = OH

221 R¹ = CH₂NHNH₂, R² = H, R³ = OH

222 R¹ = CH₂NH₂, R² = OH, R³ = H

223 R¹ = CH₂NHNH₂, R² = OH, R³ = H

224 R¹ = OH, R² = H

225 R¹ = H, R² = OH

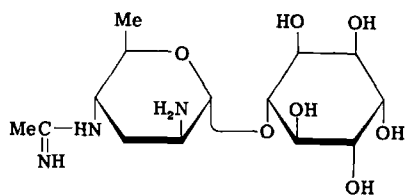
e. Kasugamycin Derivatives.—The carboxyl group of kasugamycin has been replaced by various groups.^{251,252} The 2-amino group of the kasugamine moiety was protected by formation of the dimedone derivative, and, after removal of the oxalamidine group by alkaline hydrolysis, the 4-amino group liberated was caused to react with a number of imido esters [R-C(=NH)—OEt]. Finally, removal of the dimedone group furnished the amidino derivatives of kasuganobiosamine. The methyl derivative named BL-A₂ (**226**) was found to be more active than the parent antibiotic against certain Gram-negative bacteria, including *Pseudomonas*.

f. Spectinomycin Derivatives.—Catalytic hydrogenation, or borohydride reduction, of spectinomycin gave dihydrospectinomycin (**227**), which prevents growth of various micro-organisms.²⁵³ N,N'-Bis(trifluoro-

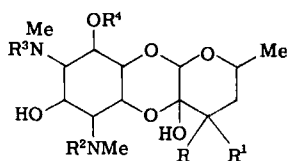
(251) M. J. Cron, R. E. Smith, I. R. Hooper, J. G. Keil, E. A. Ragan, R. H. Schreiber, G. Schwab, and J. C. Godfrey, *Antimicrob. Agents Chemother.*, 219 (1969).

(252) J. G. Keil and R. H. Schreiber, Ger. Pat. 2,121,435 (Dec. 2, 1971); *Chem. Abstr.* **76**, 113,490 (1972).

(253) H. Hoeksema and P. F. Wiley, U. S. Pat. 3,165,533 (Jan. 12, 1965); *Chem. Abstr.*, **62**, 14,689 (1965).

BL-A₂**226**

acetyl)spectinomycin (**228**) and its 9-trifluoroacetate (**229**) have antibacterial activity.²⁵⁴



227 R, R¹ = OH, H;
R² = R³ = R⁴ = H

228 R, R¹ = =O;
R² = R³ = COCF₃; R⁴ = H

229 R, R¹ = =O;
R² = R³ = R⁴ = COCF₃

(254) R. D. Birkenmeyer and H. Hoeksema, U. S. Pat. 3,184,478 (May 18, 1965); *Chem. Abstr.*, 63, 5657 (1965).

BIOCHEMICAL MECHANISM OF RESISTANCE TO AMINOGLYCOSIDIC ANTIBIOTICS

By HAMAO UMEZAWA

Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan

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I. INTRODUCTION

Among aminoglycosidic antibiotics, streptomycin, dihydrostreptomycin, kanamycin A, kanamycin B, a mixture of gentamicins C_{1a}, C₁, and C₂, a mixture of neomycins B and C, a mixture of paromomycins I and II, and ribostamycin are used for the treatment of bacterial diseases. These antibiotic substances inhibit protein synthesis on bacterial ribosomes, although the most sensitive steps inhibited are different for different antibiotics. If a strain of a bacterium is successively cultured in a medium containing one of these antibiotics, resistant clones can be readily obtained. In such resistant strains, the ribosome system gains the resistance, and protein synthesis is not inhibited by these antibiotics. It should be noted that resistant organisms appearing in patients are different in their genetic and biochemical mechanism of resistance from those artificially obtained in laboratories.

Among pathogens, resistant organisms that have been found in patients are resistant staphylococci and resistant Gram-negative organisms.

Ochiai and coworkers,¹ in 1959, and Akiba and coworkers,² in 1960, found that the resistance of dysentery bacilli can be transferred by recombination to *Escherichia coli*. These studies drew the attention of microbial geneticists, and it is now well known that the resistance of Gram-negative organisms is due to an extranuclear, genetic material, called R factor, which can be transferred by recombination among organisms of the same species and also among different species. The resistance of staphylococci that is transferred by transduction is also controlled by an extranuclear, genetic material called plasmid. Resistance of *Pseudomonas aeruginosa* has also been confirmed to be due to a kind of R factor. There are many reviews, including the one written by Mitsunashi,³ on genetic mechanism of resistance in strains isolated from patients.

In the present article, the biochemical mechanism of resistance of the strains isolated from patients is described. Before studies of the biochemical mechanism of resistance had been made, the resistance of bacteria isolated from patients was imagined to be due to some change in membrane permeability, because protein synthesis on the ribosome system was not resistant. In 1965, Okamoto and Suzuki⁴ found chloramphenicol acetyl transferase in *E. coli* carrying R factor, and in their paper, they also suggested that the same organism produces enzymes which catalyze the reaction of kanamycin with acetyl coenzyme A and the reaction of streptomycin with adenosine 5'-triphosphate. Following this work, study of the mechanism of resistance to aminoglycosidic antibiotics was initiated by H. Umezawa and coworkers.^{5,6} They first examined the reactions of kanamycin-neomycin acetyl transferase (which transfers the acetyl group of acetyl coenzyme A to the 6'-amino group of kanamycins A and B), and of kanamycin-neomycin phosphate transferase (which transfers a phosphate group from adenosine 5'-triphosphate to the 3'-hydroxyl group of kanamycins). As a result of these reactions, kanamycins are inactivated. They^{5,6} described a method that gave enough of the enzyme reaction-products for structural determination, and they

- (1) K. Ochiai, T. Totani, and Y. Toshiki, *Nippon Iji Shimpō* (Tokyo), No. 1837, 25 (1959) (in Japanese).
- (2) T. Akiba, K. Koyama, Y. Ishii, S. Kimura, and T. Fukushima, *Nippon Iji Shimpō* (Tokyo), No. 1886, 45 (1960) (in Japanese); *Jap. J. Microbiol.*, **4**, 219 (1960); *Chem. Abstr.*, **55**, 15,619e (1961).
- (3) S. Mitsunashi, "Transferable Drug Resistance Factor R," University of Tokyo Press, Tokyo, 1971.
- (4) S. Okamoto and Y. Suzuki, *Nature*, **208**, 1301 (1965).
- (5) H. Umezawa, M. Okanishi, R. Utahara, K. Maeda, and S. Kondo, *J. Antibiot.* (Tokyo), **20**, 136 (1967).
- (6) H. Umezawa, M. Okanishi, S. Kondo, K. Hamana, R. Utahara, K. Maeda, and S. Mitsunashi, *Science*, **157**, 1559 (1967).

elucidated the structures of most of the reaction products. They also proved the direct relationship between the enzymes and the resistance; this was first shown by the fact that kanamycin C (which has no 6'-amino group to be acetylated) inhibits⁷ the resistant strain that produces the enzyme involved in acetylation of the 6'-amino group of kanamycins A and B. It was further proved, more conclusively, by the synthesis of 3'-deoxykanamycin⁸ and 3',4'-dideoxykanamycin B,⁹ which do not have a hydroxyl group phosphorylated by kanamycin-neomycin phosphate transferase, and which inhibit the resistant organisms producing this enzyme.

The reaction mechanism of kanamycin-neomycin phosphate transferase has been studied in detail, and the result was sound enough to permit prediction of the structures of derivatives active in inhibiting the growth of resistant organisms. The compounds thus synthesized are proving useful in the treatment of resistant infections, and are becoming more and more valuable as probes for further analysis of the biochemical mechanism of resistance.

Determination of the structures of enzymes involved in resistance should, in the future, give information useful in predicting new types of resistant organisms that may develop, and should prove useful in predicting the structures of compounds active against future resistant strains.

II. KANAMYCIN-NEOMYCIN PHOSPHATE TRANSFERASES

1. General

A kanamycin phosphate transferase that transfers a phosphate group from adenosine 5'-triphosphate to kanamycin A was first found⁶ in the supernatant liquor of disrupted cells of *E. coli* K12-carrying-R-factor that had been centrifuged at 105,000g. It also phosphorylates the 3'-hydroxyl group of paromamine and neamine, and it was suggested that the cross resistance among kanamycins, neomycins, and paromomycins is due to this enzyme. Phosphorylation of the 3'-hydroxyl group of ribostamycin is also catalyzed by it. Similar enzymes have been found, not only in Gram-negative bacteria carrying R factor, but also in re-

- (7) M. Okanishi, S. Kondo, Y. Suzuki, S. Okamoto, and H. Umezawa, *J. Antibiot.* (Tokyo), **20**, 132 (1967).
- (8) S. Umezawa, T. Tsuchiya, R. Muto, Y. Nishimoto, and H. Umezawa, *J. Antibiot.* (Tokyo), **24**, 274 (1971).
- (9) H. Umezawa, S. Umezawa, T. Tsuchiya, and Y. Okazaki, *J. Antibiot.* (Tokyo), **24**, 485 (1971).

sistant staphylococci and *Pseudomonas aeruginosa*, and they have been confirmed to be involved in the mechanism of resistance to these antibiotics. Furthermore, the presence of two different kanamycin-neomycin phosphate transferases was confirmed in resistant *Escherichia coli*, depending on the difference of R factors^{10,11}; one does not phosphorylate butirosins, but the other phosphorylates the 3'-hydroxyl group of butirosins. The former is called kanamycin phosphate transferase I or kanamycin-neomycin phosphate transferase I, and the latter is called kanamycin phosphate transferase II or kanamycin-neomycin phosphate transferase II. In this article, the latter names are employed.

2. Kanamycin-Neomycin Phosphate Transferase I

This enzyme was first found by Umezawa and coworkers⁶ in *Escherichia coli* K12 ML1629, which was obtained by transmission of R factor from a naturally isolated, drug-resistant strain to *E. coli* K12 ML1410 resistant to nalidixic acid. The inhibitory concentrations of kanamycin A, paromomycin, and neomycin toward *E. coli* ML1629 were higher than 320 $\mu\text{g/ml}$, that of paromamine was 320 $\mu\text{g/ml}$, and that of streptomycin was 20 $\mu\text{g/ml}$. The inhibitory concentrations for *E. coli* K12 were as follows: kanamycin A, 1.25 $\mu\text{g/ml}$; paromamine, 40 $\mu\text{g/ml}$; paromomycin, 1.25 $\mu\text{g/ml}$; neomycin, 1.25 $\mu\text{g/ml}$; and streptomycin, 2.5 $\mu\text{g/ml}$.

The active enzyme solution was prepared as follows.^{6,12} The cells were collected during the last part of the logarithmic phase of growth of *E. coli* ML1629 grown in shake cultures for 7 hours at 27° in a peptone medium, and the cells were collected by centrifugation at 6,000g for 20 minutes. The sedimented cells were washed with a buffer solution [0.06 M potassium chloride, 0.01 M magnesium chloride, and 0.006 M 2-mercaptoethanol in 0.1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) hydrochloride, pH 7.8] by centrifugation. The cells were suspended in the buffer solution in a volume equal to the cell pellet, and were disrupted by passage through a French pressure-cell (1,500 kg/cm²). The suspension of the disrupted cells was centrifuged at 30,000g for 20 minutes, and the supernatant liquor was centrifuged at 100,000g for 90 minutes. The supernatant liquor thus obtained was dialyzed overnight against the buffer solution. The enzyme solution thus prepared by centrifugation at 100,000 or 105,000g is often designated S-100 or S-105 solution. All enzymes that inactivate aminoglycosidic antibiotics have been found in S-100 or S-105 solution.

An example of the reaction mixture used for testing the enzyme activity consisted of the following materials: 0.6 ml of a buffer solution, 48 mg (80 μmoles) of disodium adenosine 5'-triphosphate with the amount

- (10) M. Yagisawa, H. Yamamoto, H. Naganawa, S. Kondo, T. Takeuchi, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 748 (1972).
- (11) M. Brzezinska and J. Davies, *Antimicrob. Agents Chemother.*, **3**, 266 (1973).
- (12) M. Okanishi, S. Kondo, R. Utahara, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **21**, 13 (1968).

of sodium hydrogen carbonate necessary for dissolution, 7 mg (10 μ moles) of kanamycin A sulfate or 4.4 mg (10 μ moles) of paromamine, and 0.3 ml of the enzyme solution in a total volume adjusted to 1.0 ml with distilled water. Tris hydrochloride or 25 mM phosphate buffer (pH 7.8) can be used. The reaction mixture was incubated for 20 hours at 37°, and heated for 5 minutes at 90° to stop the reaction. The antibacterial activity was then determined by a cylinder-plate method. All of the kanamycin A and paromamine added was inactivated.

The inactivated solution was passed through a column of Amberlite IRC-50 ion-exchange resin, and eluted with 1% aqueous ammonia. Fractions that were ninhydrin-positive and contained a product conforming with inactivated ³H-kanamycin A were combined and further purified by chromatography on Dowex-1 X-2 ion-exchange resin. The crude, inactivated kanamycin A thus obtained was further purified by chromatography on Amberlite CG-50 ion-exchange resin.

The inactivated kanamycin A was converted into kanamycin A by treatment with alkaline phosphatase or by hydrolysis at 80° in 0.4 M perchloric acid adjusted to pH 4.0 with sodium hydroxide. The empirical formula of kanamycin A monophosphate was determined by elementary analysis. Hydrolysis in 6 M hydrochloric acid for 30 minutes at 100° gave 2-deoxystreptamine, 3-amino-3-deoxy-D-glucose, and 6-amino-6-deoxy-D-glucose, determined by thin-layer chromatography on silica gel G with 51:20:6:24 propyl alcohol-pyridine-acetic acid-water or 4:2:1 butyl alcohol-acetic acid-water, and by high-voltage paper-electrophoresis at 3.5 kV for 15 minutes with 3:1:36 acetic acid-formic acid-water. The consumption of 2 moles of periodate per mole, and the presence of 6-amino-6-deoxy-D-glucose in, and the absence of 3-amino-3-deoxy-D-glucose from, the hydrolyzate after the periodate oxidation indicated that the 3-hydroxyl group of the 6-amino-6-deoxy-D-glucose moiety is phosphorylated.

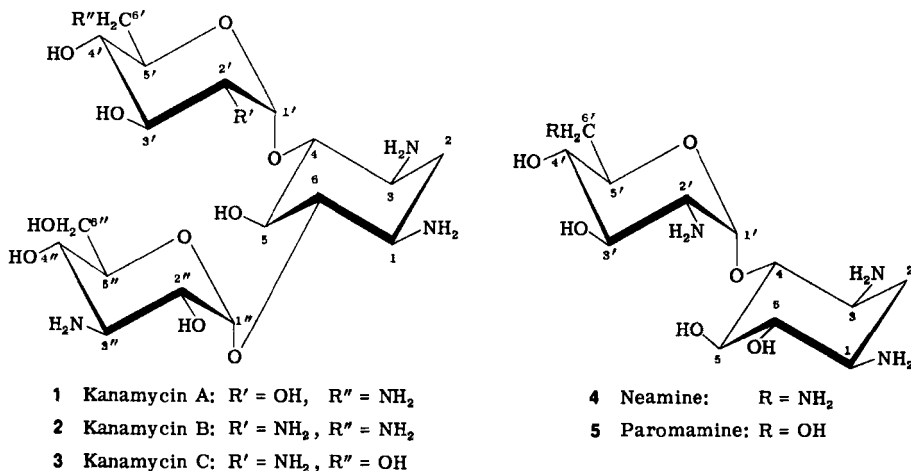
When a large amount of kanamycin was inactivated in a reaction mixture, besides kanamycin A 3'-phosphate, another product, which showed similar analytical values but which was less basic than kanamycin A 3'-phosphate, was obtained.¹³ This compound was not affected by alkaline phosphatase and phosphate diesterase. However, when kanamycin A was inactivated in dilute solution, the production of this unidentified compound was negligible.

Paromamine, obtained by methanolysis of paromomycin, was inactivated by the system used to inactivate kanamycin. After inactivation, the reaction mixture was passed through a column of Amberlite IRC-50 ion-

(13) S. Kondo, M. Okanishi, R. Utahara, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **21**, 22 (1968).

exchange resin and eluted with 1% aqueous ammonia. The ninhydrin-positive eluate was lyophilized, the product dissolved in water, and the solution chromatographed on Amberlite CG-50 resin in the ammonium form. The inactivated paromamine appeared in the aqueous effluent, and evaporation under diminished pressure, followed by addition and evaporation of methanol, gave crystalline, inactivated paromamine; it darkens at 260–290°, but does not melt at 300°. It was converted into paromamine by heating in 0.4 M perchloric acid for 20 hours at pH 4.0 and 80°, or by the action of alkaline phosphatase. An empirical formula that conformed with that calculated for paromamine monophosphate was obtained by elementary analysis. Paromamine consumed 4.4 moles of periodate per mole, and the inactivated paromamine consumed 1.6 moles of periodate per mole at pH 4.0 in 24 hours. The hydrolysis of the periodate-oxidized, inactivated paromamine gave 2-amino-2-deoxy-D-glucose, but not 2-deoxystreptamine. Thus, it was confirmed that the inactivated paromamine is paromamine 3'-phosphate.¹³

The absolute configuration of kanamycin A was determined by chemical studies, and by X-ray analysis of its monosulfate monohydrate.¹⁴ From the results obtained by X-ray analysis, the structures of the kanamycins (1, 2, and 3), neamine (4), and paromamine (5) (together with the numbering of the carbon atoms) are shown.



At first, as just described, the structures of the inactivated kanamycin A and paromamine were elucidated chemically, but they were also

(14) G. Koyama, Y. Iitaka, K. Maeda, and H. Umezawa, *Tetrahedron Lett.*, 1875 (1968).

TABLE I

Chemical Shifts and Coupling Constants for the 6-Amino-6-deoxy-D-glucose Moiety in Kanamycin A and Kanamycin A 3'-Phosphate

Compound	Chemical shift (δ)			Coupling constant (Hz)			
	H-1'	H-2'	H-3'	J _{1',2'}	J _{2',3'}	J _{3',4'}	J _{3',P}
Kanamycin A (base)	5.79	4.03	4.25	3.3	9.5	—	—
Kanamycin A 3'-phosphate (base)	5.95	4.17	4.70	3.5	10.0	8.0	8.0

elucidated solely by nuclear magnetic resonance (n.m.r.) spectroscopy at 100 MHz in deuterium oxide solution.

The n.m.r. spectrum of kanamycin base showed two signals in the anomeric region. Irradiation at δ 5.79 (J 3.3 Hz, H-1') caused complicated signals to collapse to a distinguishable doublet at δ 4.03 (J 9.5 Hz, H-2'). Irradiation at δ 4.03 confirmed that the next proton (H-3') resonated at slightly lower field than H-2'. The signal of H-3' was shown to be located at $\delta \sim 4.25$ by ^1H -[^1H] INDOR methods. Irradiation at δ 5.50 (J 3.8 Hz, H-1'') caused the complex signal to collapse to a clearly distinguishable doublet of J 10.5 Hz at δ 3.96 (H-2''). The result of irradiation at δ 3.96 suggested that the chemical shift of H-3'' to higher field was due to the effect of the amino group. Thus, it was shown that the (H-1'')-(H-2'')-(H-3'') series of signals just described are attributable to the 3-amino-3-deoxy-D-glucose moiety. Therefore, the signals of the (H-1')-(H-2')-(H-3') series must have been given by the 6-amino-6-deoxy-D-glucose moiety.¹⁵

In the n.m.r. spectrum of kanamycin A 3'-phosphate, irradiation of H-1' at δ 5.95 (J 3.5 Hz) caused the complex signals of H-2' to collapse to a sharp doublet centered at δ 4.17 (J 10.0 Hz). Irradiation at δ 4.17, attributed to H-2', caused a quartet signal centered at δ 4.70 to collapse to a triplet. Irradiation at δ 4.71 caused the signal of H-2' to collapse to a doublet (J 3.5 Hz), and signals of H-4' at δ 3.9 to change slightly. Irradiation of a signal at δ 3.90, attributed to H-4', caused the quartet signal of H-3' to collapse to a triplet ($J \sim 8$ Hz). In the spectrum of kanamycin A 3'-phosphate, the resonance position of H-3' in 6-amino-6-deoxy-D-glucose shifts by 0.45 p.p.m. to lower field than that of kanamycin A (see Table I), and the splitting pattern shows the spin-spin coupling between P and H-3' (J 8.0 Hz).¹⁵

(15) H. Naganawa, S. Kondo, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **24**, 823 (1971).

TABLE II

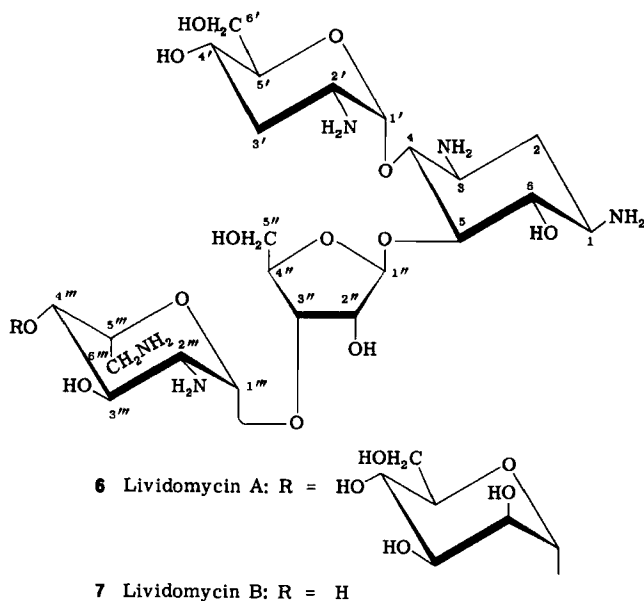
Chemical Shifts and Coupling Constants for the 2-Amino-2-deoxy-D-glucose Moiety in Paromamine and Paromamine 3'-Phosphate

Compound	Chemical shift (δ)			Coupling constant (Hz)			
	H-1'	H-2'	H-3'	J _{1',2'}	J _{2',3'}	J _{3',4'}	J _{3',P}
Paromamine (hydrochloride)	6.13	3.95	4.42	3.8	11.0	8.5	—
Paromamine 3'-phosphate (hydrochloride)	6.18	4.12	4.92	3.6	10.5	8.5	8.0
Paromamine 3'-phosphate	5.95	3.84	4.77	3.5	10.0	8.0	8.0

For paromamine 3'-phosphate (see Table II), the shift of the H-3' signal to low field (by 0.50 p.p.m.), and splitting caused by the phosphate group, are also confirmed. The resonance position of H-3' in the 2-amino-2-deoxy-D-glucose moiety of paromamine was determined by the ^1H -[^1H] INDOR method. The INDOR response (H-3') was obtained by monitoring the H-2' resonance. Thus, the H-3' resonance was confirmed as a doublet of doublets (J 11.0, 8.5 Hz) at δ 4.42. For paromamine 3'-phosphate, the H-2' signal (δ 4.12, J 3.6, 10.5 Hz) was indicated by the irradiation of the anomeric-proton signal H-1' at δ 6.18 (J 3.6 Hz). Irradiation at δ 4.92 caused signals of H-2' to collapse to a doublet (J 3.6 Hz), and signals of H-4' at the same position to simplify (J 9.5 Hz). Irradiation of H-2' and H-4' at δ 4.14 caused the multiplet of H-3' at δ 4.92 to collapse to a doublet ($J_{\text{P-O-C-H}}$ 8.0 Hz), indicating that H-3' is located on a carbon atom bearing a phosphate group. Furthermore, irradiation of the signal of ^{31}P (40.49113 MHz) caused the signal of H-3' at δ 4.92 to collapse to a doublet of doublets ($J_{2',3'}$ 10.5 Hz and $J_{3',4'}$ 8.5 Hz).¹⁵ The results obtained by n.m.r. spectroscopy indicated that the phosphate group in inactivated kanamycin A or paromamine is attached equatorially at O-3 in the respective 6-amino-6-deoxy-D-glucose or 2-amino-2-deoxy-D-glucose moieties.

Escherichia coli K12 ML1629, *E. coli* K12 ML1410 R81, and *E. coli* K12 J5 R11-2 showed similar resistant patterns to aminoglycosidic antibiotics, and the S-100 fraction contained kanamycin-neomycin phosphate transferase I. This enzyme solution also inactivated lividomycins A and B (6 and 7) in the presence of adenosine 5'-triphosphate.¹⁶ A crude powder of the inactivated lividomycin A extracted by column chroma-

(16) S. Kondo, H. Yamamoto, H. Naganawa, H. Umezawa, and S. Mitsuhashi, *J. Antibiot.* (Tokyo), **25**, 483 (1972).



tography on *O*-(carboxymethyl)Sephadex C-25 was dissolved in 0.1 *M* ammonium formate, and chromatographed on *O*-(carboxymethyl)-Sephadex C-25 equilibrated with 0.1 *M* ammonium formate. After the column had been washed with 0.1 *M* and 0.4 *M* ammonium formate, the inactivated product, which could be detected by the Rydon-Smith^{16a} and Hanes^{16b} reactions, was eluted with 0.8 *M* ammonium formate. The eluate was passed through a column of Amberlite CG-50 (NH_4^+) and the inactivated product was eluted with 0.5% ammonia. Thus, purified, inactivated lividomycin A was obtained as a white powder.¹⁶

Inactivated lividomycin A does not melt up to 210°, and has $[\alpha]_D^{20} +52.2^\circ$ (*c* 1.5, water). The empirical formula of lividomycin A monophosphate was shown by elementary analysis. It gives positive ninhydrin, Rydon-Smith, and Hanes reactions. On high-voltage paper-electrophoresis at 3.0 kV for 20 minutes, with 1:3:36 (v/v) formic acid-acetic acid-water, the inactivated lividomycin A moved 10.8 cm towards the cathode, while lividomycin A moved 12.3 cm. It showed no u.v. maximum (except end absorption). A band at 970 cm^{-1} (phosphoric ester) was observed in the i.r. spectrum. The inactivated lividomycin A consumes 6.2 moles of periodate per mole in 24 hours, as does lividomycin A (5.9 moles per

(16a) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).

(16b) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

mole). Methanolysis of the inactivated lividomycin A by refluxing in 0.4 *M* hydrogen chloride in methanol for 6 hours gave 3'-deoxyparomamine, methyl lividotriosaminide phosphate, and a small proportion of methyl lividobiosaminide phosphate.¹⁶

These results indicated that the inactivated lividomycin A is a monophosphate at either the 2- or 5-hydroxyl group of the *D*-ribose moiety in lividomycin A. Moreover, the results of n.m.r. decoupling experiments indicated that O-2 in the *D*-mannose moiety (H-2''': δ 4.43, *J* 1.5, 3.0 Hz) and O-3 in the neosamine B moiety (H-2''': δ 4.7; H-3''' δ 4.05) are not phosphorylated. The nuclear magnetic resonance spectrum of the inactivated lividomycin A in deuterium oxide (with tetramethylsilane as the external reference standard, δ = 0) showed four anomeric-proton signals, at δ 5.50 (*J* 1.5 Hz, ribose), δ 5.53 (*J* 1.5 Hz, mannose), δ 5.81 (*J* 3.0 Hz, neosamine B), and δ 5.82 (*J* 3.5 Hz, 3-deoxyparomose). These assignments of anomeric protons were made by decoupling experiments and by comparisons with the n.m.r. spectra of paromomycin, methyl lividotriosaminide, and methyl lividotriosaminide phosphate. Irradiation of the anomeric proton of the *D*-ribose moiety at δ 5.50 collapsed the signal at δ 3.55 (H-2'') to a doublet (*J* 3.0 Hz). From chemical-shift values and coupling constants, it was concluded that the 2-hydroxyl group of the *D*-ribose moiety is not phosphorylated. Irradiation of H-2'' at δ 3.55 collapsed the signal at δ 4.6–4.7. Irradiation at δ 4.7 collapsed the signal at δ 4.98 (H-4'', triplet) and that at δ 3.55 (H-2'') to a singlet and a doublet (*J* 3.0 Hz), respectively. Thus, the signals of H-3'' and H-5'' of the *D*-ribose moiety were confirmed to be present in the δ 4.6–4.7 region. As later described, the signal of H-5 of the *D*-ribosyl group in the adenylyl moiety of 3',4'-dideoxykanamycin B 2''-adenylate appeared at δ 4.6. Thus, it was determined that the primary hydroxyl group on C-5 of the *D*-ribose moiety in lividomycin A is phosphorylated.¹⁶

The structure, lividomycin 5''-phosphate, was furthermore proved by chemical synthesis.¹⁷ Penta-*N*-(benzyloxycarbonyl)lividomycin A was prepared from lividomycin A by the usual Schotten-Baumann procedure in a yield of 95%; it had m.p. 135–150° (dec.). Acetonation with 2,2-dimethoxypropane in *N,N*-dimethylformamide in the presence of *p*-toluenesulfonic acid at 110° for 4 hours afforded the tri-*O*-isopropylidene derivative of *N*-(benzyloxycarbonyl)lividomycin A in 49% yield; m.p. 129–133° (dec.). Phosphorylation of the sole primary hydroxyl group in the *D*-ribose moiety of *N*-(benzyloxycarbonyl)lividomycin A with diphenyl phosphorochloridate in dry pyridine gave penta-*N*-(benzyloxy-

(17) H. Yamamoto, S. Kondo, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 485 (1972).

carbonyl)-4',6':2''',3''':4''',6'''-tri-*O*-isopropylidenelividomycin A 5''-(diphenylphosphate) in 55% yield; m.p. 125–130°. The protecting groups were removed stepwise by catalytic hydrogenolysis with palladium black in acetic acid under atmospheric pressure, hydrolysis with 90% trifluoroacetic acid, and hydrogenolysis with platinum oxide in 50% aqueous ethanol at 4.3 atmospheres, to afford synthetic lividomycin A 5''-phosphate. The product was purified by passage through a column of Amberlite CG-50 (NH₄⁺) resin. Synthetic lividomycin A 5''-phosphate was found to be identical with inactivated lividomycin A in all respects (including the n.m.r. spectrum).

Partial purification of the lividomycin-inactivating enzyme was attempted by Mitsuhashi and coworkers,¹⁸ who briefly described their discovery that the enzyme obtained by fractionation with ammonium sulfate and column chromatography on Sephadex G-100 inactivates lividomycins A and B, but not kanamycin A, indicating involvement of two different enzymes in the phosphorylation of lividomycin and kanamycin. However, it was definitely proved by H. Umezawa and coworkers¹⁹ that kanamycin–neomycin phosphate transferase I phosphorylates the 5''-hydroxyl group of lividomycins. The observation by Mitsuhashi and coworkers¹⁸ was probably occasioned by instability of the enzyme and the higher activity of the enzyme in phosphorylating lividomycins than in phosphorylating kanamycin A.

A survey of resistant organisms isolated from patients showed the frequent occurrence of kanamycin–lividomycin cross-resistance in Gram-negative organisms and staphylococci. Umezawa and coworkers¹⁹ attempted to isolate kanamycin- and lividomycin-phosphorylating enzymes from *Escherichia coli* K12 J5 R11-2 (which produces kanamycin–neomycin phosphate transferase I). Purification of the enzyme by successive precipitation with ammonium sulfate and column chromatography on Sephadex G-100 and on DEAE-Sephadex A50 gave only a 3- to 4-fold purification, as shown by the activity per mg of protein. The enzyme was very unstable. 1,4-Dithiothreitol (10 mM) prevented denaturation, and partially restored the activity of the denatured enzyme. The kanamycin-inactivating and lividomycin-inactivating activities were always found in the same fraction during each chromatographic purification.

Kanamycin–neomycin phosphate transferase I was successfully purified¹⁹ by affinity chromatography with lividomycin A–Sephadex 4B. Livi-

(18) M. Yamaguchi, T. Koshi, F. Kobayashi, and S. Mitsuhashi, *Antimicrob. Agents Chemother.*, **2**, 142 (1972).

(19) H. Umezawa, H. Yamamoto, M. Yagisawa, S. Kondo, T. Takeuchi, and Y. A. Chabbert, *J. Antibiot. (Tokyo)*, **26**, 407 (1973).

domycin A has a primary amino group that is far from the enzyme-binding site.

Lividomycin A-Sepharose 4B was prepared as follows: cyanogen bromide-activated Sepharose 4B (9 g; Pharmacia Fine Chemicals AB, Uppsala, Sweden) was successively washed with 1.8 liters of 1 mM hydrochloric acid, 1 liter of cold water, and 50 ml of 0.1 M sodium hydrogen carbonate–0.5 M sodium chloride; then 1.0 g of lividomycin A in 45 ml of 0.1 M sodium hydrogen carbonate–0.5 M sodium chloride was added, and the mixture was stirred at 4° overnight; lividomycin A-Sepharose 4B thus prepared was treated with 300 ml of 1.0 M 2-aminoethanol hydrochloride (pH 8.0), and successively washed with 100 ml of 0.1 M acetate buffer–1.0 M sodium chloride (pH 4.0), 100 ml of 0.1 M borate buffer–1.0 M sodium chloride (pH 8.0), and distilled water. The supernatant liquor from centrifugation of disrupted cells of *E. coli* K12 J5 R11-2 at 100,000g had a kanamycin-inactivating activity of 0.58 u/mg (that is, 0.58 μ mole of kanamycin A was inactivated by 1 mg of protein per hour) and a lividomycin-inactivating activity of 2.27 u/mg.

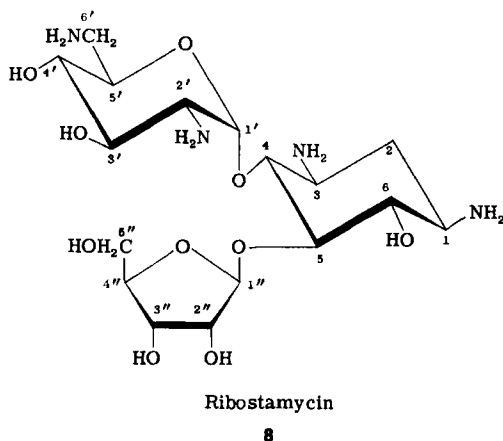
The lividomycin A-Sepharose 4B was packed in a column (1.0 \times 11.7 cm; \sim 9 ml) and washed with 20 mM Tris hydrochloride buffer containing 10 mM magnesium acetate, 60 mM potassium chloride, and 10 mM 1,4-dithiothreitol, and 5 ml of the supernatant liquor from the disrupted cells was passed through the column at the rate of 40 ml/hour. The enzyme adsorbed was successively eluted with a gradient of sodium chloride (0 to 0.8 M) in 20 mM Tris-hydrochloric acid buffer, pH 7.2. The enzyme that phosphorylates and inactivates both lividomycin A and kanamycin appeared in the eluate made with 0.4 to 0.6 M sodium chloride. By this procedure, the enzyme was purified 25- to 40-fold in phosphorylating activity for both kanamycin A and lividomycin A. The purified fraction obtained showed two bands in disc-gel electrophoresis, and one of the bands had the activity as regards phosphorylating kanamycin A and lividomycin A.

On using the enzyme purified by the affinity chromatography just described (a kanamycin-inactivating activity of 13.2 u/mg, and a lividomycin A-inactivating activity of 62.5 u/mg), kanamycin inhibited the phosphorylation of lividomycin A, and lividomycin A inhibited the phosphorylation of kanamycin.¹⁹ After the reaction, these residual antibiotics were determined by high-pressure, liquid chromatography.¹⁹ First, these antibiotics in the reaction mixture were adsorbed on a column (1.0 ml) of Amberlite CG-50 (NH_4^+) ion-exchange resin and eluted with 4.0 ml of 2 M aqueous ammonia, and the eluate was passed through a column (0.5 ml) of Dowex-1 X-2 (OH^-) resin, and freeze-dried to a powder under vacuum. The powder thus obtained was dissolved in 0.14 to 0.18 ml of distilled water, and the solution was subjected to high-pressure, liquid chromatography (Varian LC4200, under a pressure of 3,350 lb.in.⁻², at 50°, at a flow-rate of 15 ml of water/hour) on a column (0.2 \times 100 cm) of Aminex A-28 (OH^-) ion-exchange resin by injecting 10 μ l of the solution of the powder. Kinetic studies showed the competitive inhibition of phosphorylation of lividomycin A by kanamycin A. The reaction products of kanamycin-neomycin phosphate transferase I

(such as neamine 3'-phosphate and kanamycin 3'-phosphate) also inhibit phosphorylation of lividomycin A.¹⁹

The appearance of kanamycin and lividomycin A phosphorylating activities in the same peak in the chromatography, and the mutual, competitive inhibition of the phosphorylation by these antibiotics, constitute enough proof of the involvement of a single enzyme in the phosphorylation of kanamycin and lividomycin A.

Ribostamycin (8) contains two hydroxyl groups (the 3-hydroxyl group



of the 2,6-diamino-2,6-dideoxy-D-glucose moiety and the 5-hydroxyl group of the D-ribose moiety) which can be phosphorylated by kanamycin-neomycin phosphate transferase I. A molecular model in which these hydroxyl groups are close to each other can readily be built. Therefore, in the enzyme reaction, the terminal phosphorus atom of adenosine 5'-triphosphate is considered to be located close to both hydroxyl groups. It may be closer to the 3'-hydroxyl group, because the reaction product of ribostamycin is ribostamycin 3'-phosphate, but the enzyme reaction on 3',4'-dideoxyribostamycin²⁰ (which lacks the 3'-hydroxyl group) gave 3',4'-dideoxyribostamycin 5''-phosphate. Confirming this fact, strains such as *Escherichia coli* K12 ML1629, *E. coli* K12 ML1410 R81, and *E. coli* K12 J5 R11-2 carrying R factor producing kanamycin-neomycin phosphate transferase I are resistant to ribostamycin and 3',4'-dideoxyribostamycin. Kanamycin-neomycin phosphate transferase I requires magnesium ion for the reaction. The optimal pH value is ~ 7.2 . As the

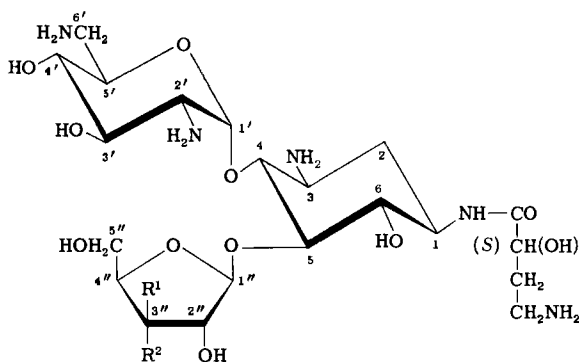
(20) S. Umezawa, T. Tsuchiya, D. Ikeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 613 (1972).

3'-hydroxyl groups of paromamine and neamine are phosphorylated, the 3-amino-3-deoxy-D-glucose moiety in kanamycins is not involved in binding of these antibiotics with kanamycin phosphate transferase I.

Neither 6-amino-6-deoxy-D-glucose nor its methyl α -D-glycoside undergoes the enzyme reaction.^{20a} D-Glucose does not inhibit the enzyme reaction, but methyl 6-amino-6-deoxy-D-glucoside and methyl 3-amino-3-deoxy- β -D-glucoside cause significant inhibition. These requirements, of (a) the substrate structures that undergo the enzyme reaction, and (b) the structures of inhibitors, are similar to those which have been studied in kanamycin-neomycin phosphate transferase of *Pseudomonas aeruginosa*, as described later (see p. 201).

3. Kanamycin-Neomycin Phosphate Transferase II

Butirosins A (9) and B (10), which have been found by Woo and co-



9 Butirosin A: $R^1 = \text{OH}$; $R^2 = \text{H}$

10 Butirosin B: $R^1 = \text{H}$; $R^2 = \text{OH}$

workers²¹ in *Bacillus circulans*, have interesting structures. Butirosin B, 1-N-(L-4-amino-2-hydroxybutyryl)ribostamycin, has been synthesized from ribostamycin.²² Butirosins inhibited *E. coli* carrying R factor, which produced kanamycin-neomycin phosphate transferase I and was not phosphorylated by this enzyme. Thus, two possibilities are suggested: either that the 1-amino group of the 2-deoxystreptamine moiety of ribostamycin is involved in binding with kanamycin-neomycin phosphate transferase I, or the L-4-amino-2-hydroxybutyryl group on the 1-amino

(20a) H. Umezawa, unpublished results.

(21) P. W. K. Woo, H. W. Dion, and Q. R. Bartz, *Tetrahedron Lett.*, 2625 (1971).

(22) D. Ikeda, T. Tsuchiya, S. Umezawa, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 741 (1972).

group causes steric hindrance to binding of the other groups with this enzyme.

Independently, Umezawa and coworkers¹⁰ and Brzezinska and Davies¹¹ found another enzyme, designated kanamycin phosphate transferase II or kanamycin-neomycin phosphate transferase II, in *E. coli* carrying R factor, and it was found by the former authors¹⁰ that this enzyme also catalyzes the transfer of a phosphate group from adenosine 5'-triphosphate to the 3'-hydroxyl group of the kanamycins, paromamine, neamine, neomycin, ribostamycin, and the butirosins. Therefore, kanamycin-neomycin phosphate transferase II differs from I in the following points: II phosphorylates butirosin, but not lividomycin, whereas I phosphorylates the latter, but not the former.

The cells of *E. coli* K12 JR66/W677 were homogenized, and the S-100 fraction (the supernatant liquor from centrifugation at 100,000g) was prepared as already described (see p. 186). Butirosin A (100 mg) was inactivated at 37° for 1 hour in a reaction mixture containing 12.5 mg of S-100 protein, 800 μ moles of disodium adenosine 5'-triphosphate in 5 ml of 0.1 M sodium hydrogen carbonate, 20 ml of 1 M potassium phosphate buffer (pH 7.8), and 20 ml of 100 mM magnesium acetate in 60 mM 2-mercaptoethanol, the total volume being made to 200 ml with distilled water. After the reaction, 200 ml of distilled water was added, and the mixture was heated in a boiling-water bath for 10 minutes to stop the reaction. The suspension was filtered, and the filtrate was passed through a column (50 ml) of Amberlite CG-50 (NH₄⁺) ion-exchange resin. The column was washed with 1 liter of distilled water, and the inactivated butirosin A was eluted with 0.1 M aqueous ammonia. The eluate (which gave positive ninhydrin and Rydon-Smith reactions) was collected, and evaporated to dryness, yielding 101 mg of white powder. The powder was subjected to rechromatography on a column (50 ml) of Amberlite CG-50 (NH₄⁺) ion-exchange resin and eluted with 0.05 M aqueous ammonia. Purified, inactivated butirosin A (89 mg) was obtained as a white powder.¹⁰

The inactivated butirosin A darkens at 217–221°. The empirical formula of butirosin A monophosphate monohydrate was determined by elementary analysis. It gave positive ninhydrin, Rydon-Smith, and Hanes reactions. On high-voltage, paper electrophoresis under 3.5 kV for 15 minutes with 1:3:36 (v/v/v) formic acid-acetic acid-water, the inactivated butirosin A moved 14.6 cm towards the cathode, whereas butirosin A moved 19.0 cm. It showed only end absorption in its u.v. spectrum, and a band at 960 cm⁻¹ (phosphoric ester) in the i.r. spectrum. Hydrolysis by alkaline phosphatase yielded butirosin A.

The n.m.r. spectrum of the inactivated butirosin A in deuterium oxide, with tetramethylsilane as the external reference standard ($\delta = 0$), showed signals of the 4-amino-2-hydroxybutyryl moiety at δ 4.78 (*J* 4.0 and 7.5 Hz, quartet, α -methine), 3.63 (*J* 7.5 Hz, triplet, γ -methylene) and \sim 2.5 (multiplet, β -methylene). The chemical shifts of these signals were the

same as for butirosin A. Irradiation at δ 6.40 (H-1') caused the signal at δ 3.62 (H-2') to collapse to a doublet ($J_{2',3'}$ 10.0 Hz). Irradiation at δ 3.62 indicated the H-3' signal resonating at δ 4.5. The chemical shift of H-3' was similar to that (δ 4.49) of H-3' of ribostamycin 3'-phosphate. Thus, it was evident that the phosphate group is attached to the 3'-hydroxyl group of the 2,6-diamino-2,6-dideoxy-D-glucose moiety.¹⁰ The result was confirmed by an n.m.r.-spectroscopic study of the methanolysis product of the inactivated butirosin A. Refluxing of the inactivated butirosin A with 0.4 M hydrogen chloride in methanol for 2 hours gave 1-N-(L-4-amino-2-hydroxybutyryl)neamine 3'-phosphate. Its n.m.r. spectrum showed the signal of the anomeric proton (H-1') at δ 6.02. A spin-decoupling experiment indicated the signals of H-2' at δ 3.61 ($J_{1',2'}$ 3.5 Hz, $J_{2',3'}$ 10.0 Hz) and H-3' at δ 4.66 ($J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 9.0 Hz, $J_{3',P}$ 8.5 Hz). From the value of the chemical shift and splitting pattern of H-3', the methanolysis product was determined to be 1-N-(L-4-amino-2-hydroxybutyryl)neamine 3'-phosphate.

The enzyme in the S-100 solution of *Escherichia coli* K12 JR66/W677 was precipitated by 30–50% saturation with ammonium sulfate, and chromatographed on a column of Sephadex G-100. The active fraction thus obtained was subjected to column chromatography on DEAE-Sephadex A-50 with a gradient of potassium chloride from 0 to 0.5 M. The enzyme activity was detected in the fraction obtained with 0.28–0.3 M eluant, and the active fraction was again subjected to column chromatography on DEAE-Sephadex A-50. The yield was 18%, and a 60-fold purification was obtained. The phosphorylating activity for both kanamycin A and butirosin A appeared in the same fraction, indicating that a single enzyme phosphorylates both antibiotics. Purification of the enzyme by affinity chromatography with 3',4'-dideoxykanamycin B-Sepharose 4B and a gradient of sodium chloride from 0 to 1.0 M was found very useful.^{20a} Application of this method to S-105 solution gave a 60-fold purification in one step, and the yield was 35%. Kanamycin-neomycin phosphate transferase II appeared in the fraction obtained with 0.5 M sodium chloride as the eluant. Streptomycin-inactivating enzyme in the S-105 solution appeared in the fraction eluted with 0.05 to 0.1 M sodium chloride, and was readily separated from the kanamycin-neomycin phosphate transferase II.

Kanamycin-neomycin phosphate transferase II also requires magnesium ion for reaction,^{20a} and its optimal pH is 6.6–7.5. It is more stable to heat than kanamycin-neomycin phosphate transferase I. It phosphorylates all of the kanamycins, 4-O-(6-amino-6-deoxy- α -D-glucopyranosyl)-6-O- α -D-glucopyranosyl-2-deoxystreptamine, 4-O-(6-amino-6-deoxy-

α -D-glucopyranosyl)-2-deoxystreptamine, neamine, paromamine, neomycin, paromomycin, ribostamycin, butirosin A, and butirosin B. Thus, the 3-amino-3-deoxy-D-glucose moiety in the kanamycins and the D-ribose moiety in the butirosins are not involved in the reaction. Similar structural requirements for inhibitors of kanamycin-neomycin phosphate transferase of *Pseudomonas aeruginosa*, as later described (see p. 201), were also observed for kanamycin-neomycin phosphate transferase II. Phosphorylation of butirosin A was competitively inhibited by one tenth the concentration of 3',4'-dideoxykanamycin B.

The involvement of kanamycin-neomycin phosphate transferase II in the mechanism of resistance was demonstrated by the fact that *Escherichia coli* K12 JR66/W677 is inhibited by 3',4'-dideoxybutirosin B,²³ 3',4'-dideoxyribostamycin,²⁰ 3',4'-dideoxyneamine,²⁴ and lividomycins that do not undergo this enzyme reaction.

4. Kanamycin-Neomycin Phosphate Transferase in *Pseudomonas aeruginosa*

Most strains of *Pseudomonas aeruginosa* are resistant to various antibiotics, and the natural resistance, at least to aminoglycosidic antibiotics, has been confirmed as being related to formation of enzymes that inactivate these antibiotics. The presence of such an enzyme in *Pseudomonas aeruginosa* was first reported by Umezawa and coworkers.²⁵

Pseudomonas aeruginosa strain H₀ was grown in 0.3% D-glucose broth at 37°, the cells were harvested in the late logarithmic phase, and the enzyme solution (S-105 solution) was prepared by centrifugation of the disrupted cells at 105,000g as already described (see p. 186). Kanamycin A was inactivated at 30° for 20 hours in a reaction mixture consisting of S-105 solution (4 mg of protein/ml), kanamycin A sulfate (10 mM), adenosine 5'-triphosphate (20 mM), potassium chloride (156 mM), magnesium acetate (20 mM), 2-mercaptoethanol (15.6 mM), and Tris hydrochloride (260 mM), pH 7.5. The reaction product was extracted and purified by a method similar to that used for isolation of kanamycin A 3'-phosphate (see p. 187). The identity of the inactivated product with kanamycin A 3'-phosphate was confirmed by their having the same behavior in high-voltage, paper electrophoresis and in thin-layer chromatography with 25:10:3:12 (v/v) propyl alcohol-pyridine-acetic acid-water, by conversion into kanamycin A in 0.4 M perchloric acid (adjusted to

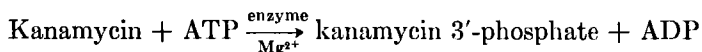
- (23) D. Ikeda, T. Tsuchiya, S. Umezawa, H. Umezawa, and M. Hamada, *J. Antibiot.* (Tokyo), **26**, 307 (1973).
- (24) S. Umezawa, T. Tsuchiya, T. Jikihara, and H. Umezawa, *J. Antibiot.* (Tokyo), **24**, 711 (1971).
- (25) H. Umezawa, O. Doi, M. Ogura, S. Kondo, and N. Tanaka, *J. Antibiot.* (Tokyo), **21**, 154 (1968).

pH 4.0) at 80–83°, and by the presence of 6-amino-6-deoxy-D-glucose in the hydrolyzate of the periodate-oxidized material.

Kanamycin–neomycin phosphate transferase in this strain was partially purified by Umezawa and coworkers.^{26,27}

All of the procedures were conducted below 4°. To the S-105 fraction [4.0 absorbance units; one absorbance unit is that of the amount of protein existing in 1 ml of a solution whose absorbance (1-cm light-path) at 280 nm is 1.0] was added ammonium sulfate to achieve 40% saturation. The precipitate was collected by centrifugation, dissolved in a buffer (60 mM potassium chloride, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol, and 100 mM Tris hydrochloride, pH 7.8), and dialyzed against the same buffer. The dialyzed solution was applied to a column of Sephadex G-100, and this was developed with the same buffer. The effluent was collected in 10-ml fractions. The fractions containing the kanamycin-phosphorylating enzyme were combined, applied to a column of DEAE-Sephadex A-50, and fractionated by elution with a linear gradient of potassium chloride (from 0.1 to 0.5 M in the buffer already described). Active fractions were combined, and column chromatography on Sephadex G-100 and DEAE-Sephadex A-50 was repeated. A final purification of 360-fold was achieved.²⁷

By use of the most-purified enzyme, it was confirmed that kanamycin A is completely phosphorylated in the presence of an equimolar amount of adenosine 5'-triphosphate, which is converted into adenosine 5'-pyrophosphate, as follows.



Guanosine 5'-triphosphate, cytidine 5'-triphosphate, and uridine 5'-pyrophosphate cannot replace adenosine 5'-triphosphate. Magnesium ion is required for this reaction; it may be replaced by manganese, zinc, and cobalt divalent ions. The reaction rate is increased when the temperature is raised from 30 to 55°, but, at 65°, the reaction proceeds only during the first 10 minutes, and, at 75°, no reaction occurs. The optimal pH is 6.5–7.5. Compounds that interact with the sulfhydryl group inhibit the reaction; *p*-chloromercuribenzoate shows 100% inhibition at 0.5 mM.

The substrate specificity of this enzyme was examined by using a reaction mixture consisting of 10 mM adenosine 5'-triphosphate, 20 mM potassium chloride, 5 mM magnesium acetate, 0.013 absorbance unit of the enzyme, a substrate (4 mM), and 10 mM Tris hydrochloride (made to pH 7.5 with hydrochloric acid). The reaction was performed for 1 hour

(26) O. Doi, M. Ogura, N. Tanaka, and H. Umezawa, *Appl. Microbiol.*, **16**, 1276 (1968).

(27) O. Doi, S. Kondo, N. Tanaka, and H. Umezawa, *J. Antibiot. (Tokyo)*, **22**, 273 (1969).

at 37°. Kanamycin A, kanamycin B, kanamycin C, kanamycin 6''-phosphate, 3''-deamino-3''-hydroxykanamycin, neamine, paromamine, and 4-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine were completely phosphorylated. Therefore, the 3-amino-3-deoxy-D-glucose moiety does not influence the specificity of the substrate. Neomycin B and paromomycin were completely inactivated. Kanamycin 6'-acetamide was phosphorylated slightly.²⁷

The data obtained regarding the substrate specificity indicated that the enzyme reaction does not require the whole molecule of kanamycins, but only the disaccharide moiety; that is, 4-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-2-deoxystreptamine, neamine, or paromamine are sufficient for the enzyme reaction to occur. D-Glucose, 2-amino-2-deoxy-D-glucose, and 6-amino-6-deoxy-D-glucose and its methyl α -D-glucopyranoside are not phosphorylated, and, therefore, the 2-deoxystreptamine moiety appears necessary for the enzyme reaction.

In a reaction mixture containing 0.2 mM kanamycin, 5 mM adenosine 5'-triphosphate, 0.001 absorbance unit/0.5 ml of the enzyme, 10 mM magnesium acetate, 60 mM potassium chloride, and 100 mM Tris hydrochloride, pH 7.5, inhibition by compounds structurally related to kanamycins has been examined. The inhibitors were added at a concentration of 0.8 or 3.2 mM. D-Glucose, methyl α -D-glucopyranoside, and 2-amino-2-deoxy-D-glucose showed no inhibition, but methyl 2-amino-2-deoxy- β -D-glucopyranoside showed 30% inhibition at 3.2 mM; this result suggested that the amino group on C-2' of the D-glucopyranoside binds with the enzyme. 6-Amino-6-deoxy-D-glucose showed 18% inhibition at 3.2 mM, and its methyl α -D-glucopyranoside showed 22% inhibition at 3.2 mM; this result suggested that the amino group on C-6' of the D-glucopyranoside binds with the enzyme. Kanamycin 6'-acetamide also showed 35% inhibition at 3.2 mM. 3-Amino-3-deoxy-D-glucose showed strong inhibition (that is, 30% at 3.2 mM). Its methyl α -D-glucopyranoside showed stronger inhibition (67% at 3.2 mM). Inhibition by 3-amino-3-deoxy-D-glucose and its methyl α -D-glucopyranoside was competitive with kanamycin A. These results suggested the involvement of the 2'-amino, 6'-amino, and 3'-hydroxyl groups in binding with the enzyme. 2-Deoxystreptamine showed no inhibition at 3.2 mM.

Compounds structurally related to adenosine 5'-triphosphate also exhibit inhibition. Among adenosine 5'-pyrophosphate, adenosine 5'-phosphate, adenosine, and adenine, adenosine showed the strongest inhibition. This result indicated that the adenine and D-ribose moieties of adenosine 5'-triphosphate bind with the enzyme. Formycin A is an analog of adenosine, and exhibits inhibition (61% at 3.2 mM); formycin B is less active. This suggested that the amino group of adenosine binds with the

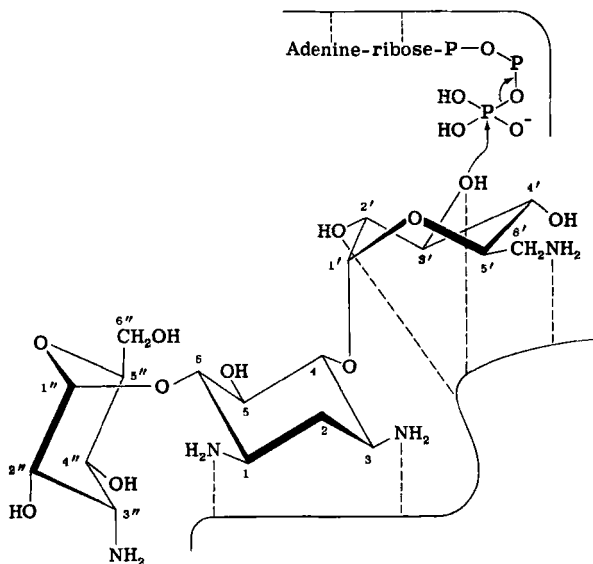


FIG. 1.—Reaction of Kanamycin–Neomycin Phosphate Transferase in *Pseudomonas aeruginosa*, Transferring the Terminal Phosphate Group of Adenosine 5'-Triphosphate to the 3'-Hydroxyl Group of Kanamycin A.

enzyme. Inhibition by formycin A is competitive with adenosine 5'-triphosphate.²⁷

The enzyme reactions can, therefore be shown²⁸ schematically as in Fig. 1. For phosphorylation of the 3'-hydroxyl group of kanamycins, it is necessary that this hydroxyl group be brought close to the terminal phosphorus atom of adenosine 5'-triphosphate. Moreover, this phosphorus atom must gain a positive charge. The binding of 6-amino-6-deoxy-D-glucose, 2-amino-2-deoxy-D-glucose, or 2,6-diamino-2,6-dideoxy-D-glucose moieties of kanamycins with the enzyme must bring the 3'-hydroxyl group close to the terminal phosphorus atom of adenosine 5'-triphosphate. The binding of adenosine 5'-triphosphate with the enzyme, in which the adenosine moiety plays the most important role, is also helpful in bringing the terminal phosphorus atom close to the hydroxyl group. Magnesium ion may play a role, either to increase the positive charge of the phosphorus atom, or to bring the reactive groups close together. The reaction products, adenosine 5'-pyrophosphate and the 3'-phosphates

- (28) H. Umezawa, in "Progress in Antimicrobial and Anticancer Chemotherapy (Proceedings of the 6th International Congress of Chemotherapy)," University of Tokyo Press, Tokyo, 1970, Vol. 2, p. 566.

of kanamycins, have less affinity for the enzyme than for the substrates, and are replaced by adenosine 5'-triphosphate and the kanamycins. The enzyme has no adenosine 5'-triphosphatase activity.

The information on the mechanism of the reaction just described is useful in predicting structures possibly resistant to enzymic phosphorylation. Another useful point stemming from a knowledge of the mechanism of the reaction is in finding inhibitors of this reaction. As already described, 3-amino-3-deoxy-D-glucose and its methyl α -D-glycopyranoside can inhibit the enzyme reaction. Although it is weak, this inhibitor increases the antibacterial effect of kanamycin A against *Pseudomonas aeruginosa* and also against *Escherichia coli* carrying R factor producing the phosphorylating enzyme.

The strain of *Pseudomonas aeruginosa* H₉ is not resistant to lividomycins, and kanamycin-neomycin phosphate transferase obtained from this strain is more stable than kanamycin-neomycin phosphate transferase I. Therefore, kanamycin-neomycin phosphate transferase of *Ps. aeruginosa* H₉ is more closely related to kanamycin-neomycin phosphate transferase II in *E. coli* carrying R factor, as already described (see p. 196). A similar kanamycin-neomycin phosphate transferase was also observed²⁹ in another strain, *Ps. aeruginosa* TK-157.

The supernatant liquor of disrupted cells of *Pseudomonas aeruginosa* TI-13 inactivates kanamycin and lividomycins in the presence of adenosine 5'-triphosphate.³⁰ The inactivated lividomycin A was confirmed¹⁸ to be lividomycin A 5''-phosphate. This strain was resistant both to the kanamycins and the lividomycins. When S-105 solution of *Pseudomonas aeruginosa* TI-13 was subjected to affinity chromatography on lividomycin A-Sepharose 4B, kanamycin-inactivating activity appeared in the effluent in 20 mM Tris hydrochloride, pH 7.2, and lividomycin-inactivating activity appeared in the fraction with 0.1 to 0.5 M sodium chloride in the same buffer.^{20a} When the S-105 solution was subjected to affinity chromatography on kanamycin A-Sepharose 4B, the lividomycin-inactivating activity appeared in the buffer, and the kanamycin-inactivating activity appeared in the fraction with 0.1 M sodium chloride in the buffer. The kanamycin phosphate transferase obtained in this way inactivated butirosin A and, therefore, it was of the type of kanamycin-neomycin phosphate transferase II. The behavior of the lividomycin-inactivating enzyme in the chromatography suggested that it differed

(29) F. Kobayashi, M. Yamaguchi, and S. Mitsuhashi, *Japan. J. Microbiol.*, **15**, 285 (1971).

(30) F. Kobayashi, M. Yamaguchi, and S. Mitsuhashi, *Antimicrob. Agents Chemother.*, **1**, 17 (1972).

from kanamycin-neomycin phosphate transferase I, and was specific in the phosphorylation of the 5''-hydroxyl group of lividomycin.

Phosphorylation of the 3'-hydroxyl group of paromamine has been also reported³¹ for the enzyme extracted from *Pseudomonas aeruginosa* NIHJ B-328.

5. Kanamycin-Neomycin Phosphate Transferase in *Staphylococci*

Resistance of staphylococci to kanamycin has been shown to be caused by a kanamycin-neomycin phosphate transferase. Two multiple, drug-resistant strains, B 294 and B 295, of staphylococci were grown in 0.3% D-glucose broth at 37°. The cells were harvested at the logarithmic phase of growth (3-hour culture) by centrifugation at 10,800g for 30 minutes, and were then washed, ground with quartz sand in a buffer solution, and further disrupted by repeated freezing and thawing. The extract was centrifuged at 4,000g for 20 minutes, and the supernatant liquor contained a kanamycin-inactivating enzyme. The optimal pH for the reaction was approximately 7.0 to 7.5. The inactivated kanamycin A was extracted by procedures already described (see p. 187), and its identity with kanamycin A 3'-phosphate was confirmed.³²

Cross-resistance between kanamycins and lividomycins is frequently observed in staphylococci, suggesting the presence of kanamycin-neomycin phosphate transferase I. However, phosphorylation of butirosins or lividomycins by a staphylococcus enzyme has not yet been studied.

III. GENTAMICIN-KANAMYCIN NUCLEOTIDYL TRANSFERASE

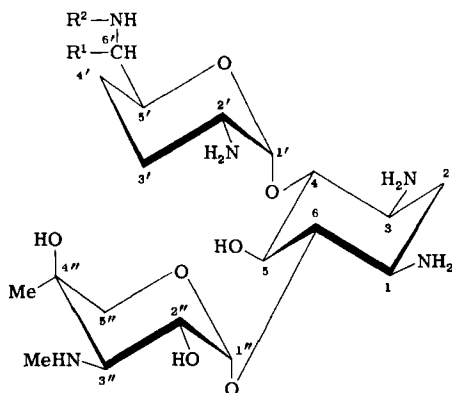
Benveniste and Davies³³ transferred R factor of a resistant strain of *Klebsiella pneumoniae* to a mutant (*Escherichia coli* W677) of *E. coli*, and obtained a resistant strain designated *E. coli* K12 JR66/W677. In this organism, these authors found gentamicin-kanamycin adenylyl transferase, which catalyzes the adenylylation of gentamicins C₁ (12), C_{1a} (11), C₂ (13), and kanamycins. The adenylylation of gentamicins was detected by an assay involving binding to cellulose phosphate paper, as follows.

The reaction mixture contained the enzyme solution, 20 nmoles of ¹⁴C-adenosine 5'-triphosphate or adenosine α -³²P-triphosphate (specific activity, 4 μ Ci/ μ mole), 10

(31) K. Maeda, S. Kondo, M. Okanishi, R. Utahara, and H. Umezawa, *J. Antibiot.* (Tokyo), **21**, 458 (1968).

(32) O. Doi, M. Miyamoto, N. Tanaka, and H. Umezawa, *Appl. Microbiol.*, **16**, 1282 (1968).

(33) R. Benveniste and J. Davies, *FEBS Lett.*, **14**, 293 (1971).



11 Gentamicin C_{1a}: R¹ = R² = H

12 Gentamicin C₁: R¹ = R² = Me

13 Gentamicin C₂: R¹ = Me, R² = H

nmoles of an aminoglycosidic antibiotic, 2.5 μ moles of Tris hydrochloride (pH 8.1 at 30°), 0.4 μ mole of magnesium chloride, and 50 nmoles of 1,4-dithiothreitol in a total volume of 55 μ l. Incubation was conducted at 30° for various periods of time, and then an aliquot (10 μ l) was pipetted onto a square (0.75 \times 0.75 cm) of cellulose phosphate paper (Whatman P-81). The squares were allowed to stand for 15 seconds to absorb the basic antibiotic, and were then immersed in hot, distilled water (70 to 80°) for 2 minutes, to stop the reaction and remove any radioactivity not bound to the antibiotic. They were then washed several times with large volumes of distilled water, dried, and counted in a scintillation counter. Control reactions for nonspecific binding of ¹⁴C-adenosine 5'-triphosphate to the paper were performed in the absence of (a) the enzyme or (b) the antibiotic.

For releasing and extracting intracellular enzymes from exponentially growing cells of *E. coli*, Nossal and Heppel³⁴ developed a method based on osmotic shock.

The cells of *E. coli* K12 JR66/W677 were washed twice at room temperature with 10 ml of 0.01 M Tris and 0.03 M sodium chloride, pH 7.3. The pellet was suspended in 10 ml of a 20% sucrose solution containing 3 mM (ethylenedinitrilo)-tetraacetic acid, and 33 mM Tris hydrochloride (pH 7.3), stirred for 10 minutes at room temperature, and centrifuged at 16,000g for 5 minutes. The pellet was then suspended in 2 ml of cold 0.5 mM magnesium chloride, and stirred for 10 minutes at 2°. The supernatant liquor from centrifugation at 26,000g for 10 minutes, which was referred to as "the osmotic shockate," was used as the enzyme solution.³⁵

LeGoffic and Chevereau³⁵ found a similar enzyme in *E. coli* K12 carrying R factor of *Klebsiella pneumoniae* origin, and they confirmed the ab-

(34) N. G. Nossal and L. A. Heppel, *J. Biol. Chem.*, **241**, 3055 (1966).

(35) F. LeGoffic and M. Chevereau, *Compt. Rend.*, **274**, 535 (1972).

sorption at 261 nm of the inactivated product. Mitsuhashi and coworkers³⁶ reported that the S-105 fraction of *K. pneumoniae* 3020 and 3694 and *E. coli* ML1410 R100⁺ adenylylates gentamicins C_{1a}, C₁, and C₂. The structure of the inactivated product was disclosed by Umezawa and coworkers³⁷ and Naganawa and coworkers³⁸ in a study of the adenylylation of 3',4'-dideoxykanamycin B^o by the S-100 solution prepared from *E. coli* K12 JR66/W677.

The inactivated 3',4'-dideoxykanamycin B in the reaction mixture was extracted by two repetitions of chromatography on Amberlite CG-50 (NH₄⁺) resin, with 0.2% ammonia for elution, and the product purified by chromatography on CM-Sephadex C-25 with ammonium formate. The inactivated product was eluted with 0.8 M ammonium formate, and separated from the salt by chromatography on Amberlite CG-50 resin with 0.2% ammonia. The inactivated product thus purified darkens at 205–209°, but does not melt even at 280°. The empirical formula of monoadenylyl-3',4'-dideoxykanamycin B trihydrate was shown by elementary analysis. It gave positive ninhydrin, Rydon-Smith,^{16a} and Hanes^{16b} reactions. On high-voltage paper-electrophoresis at 3.5 kV for 15 minutes, with 3:1:36 acetic acid–formic acid–water, the inactivated product moved 13.4 cm towards the cathode, whereas 3',4'-dideoxykanamycin B moved 16.8 cm. The inactivated product showed a u.v. maximum at 260 nm (ϵ_{mM} 15.40) in water, and a maximum at 258 nm (ϵ_{mM} 14.40) in 0.1 M hydrochloric acid. The inactivated product was hydrolyzed to 3',4'-dideoxykanamycin B and adenylic acid by snake-venom phosphate diesterase.

Nuclear magnetic resonance analysis with double and triple resonance was used to elucidate³⁸ the structure as 3',4'-dideoxykanamycin B 2''-adenylate. The spectrum of the inactivated 3',4'-dideoxykanamycin B in deuterium oxide at pH 8.0, with tetramethylsilane as the external reference standard ($\delta = 0$), showed signals at δ 8.63 and 8.85 attributable to the adenine-ring protons, and at δ 6.53, 5.28, 4.98, 4.83 and \sim 4.6 (H-2); the latter were assigned to the D-ribose-ring protons by successive double-resonance experiments, and by comparison with disodium 5'-adenylate in deuterium oxide. Therefore, these observations confirmed the presence of one molecular proportion of 5'-adenylic acid in the molecule. Irradiation at δ 5.45 (*J* 3.6, H-1'') caused the complex signal at δ 4.3 (H-2'')

- (36) F. Kobayashi, M. Yamaguchi, J. Eda, F. Higashi, and S. Mitsuhashi, *J. Antibiot.* (Tokyo), **24**, 719 (1971).
- (37) M. Yagisawa, H. Naganawa, S. Kondo, M. Hamada, T. Takeuchi, and H. Umezawa, *J. Antibiot.* (Tokyo), **24**, 911 (1971).
- (38) H. Naganawa, M. Yagisawa, S. Kondo, T. Takeuchi, and H. Umezawa, *J. Antibiot.* (Tokyo), **24**, 913 (1971).

to collapse to a triplet, whereas irradiation at δ 3.49 (H-3'') caused the same region (H-2'') to collapse to a broad doublet. Irradiation at δ 5.45 and 3.49 (triple resonance) collapsed the same position (H-2'') to a doublet (J 8.0). Irradiation at δ 4.32 (H-2'') caused the signals at δ 5.45 (H-1'') and δ 3.50 (H-3'') to collapse to a singlet and doublet (J 10.0), respectively. The high-field chemical shift of H-3'' was attributed to the amino group. These results indicated that the H-1'', H-2'', and H-3'' signals described are attributable to the 3-amino-3-deoxy-D-glucose moiety. For 3',4'-dideoxykanamycin B base in deuterium oxide, the H-1'', H-2'', and H-3'' signals of the 3-amino-3-deoxy-D-glucose moiety were observed at δ 5.52, 3.98, and 3.46, respectively. The H-2'' signal of inactivated 3',4'-dideoxykanamycin B was shifted 0.34 p.p.m. to lower field compared with 3',4'-dideoxykanamycin B. The lower-field shift indicated that the H-2'' signal is affected by the phosphoric ester function. Similar shifts were observed in the spectra of phosphorylated kanamycin, paromamine, and dihydrostreptomycin. Irradiation of the H-1'', H-3'', and ^{31}P (40.489023 MHz) signals collapsed the complicated signal of H-2'' to a singlet. This quadruple-resonance result conclusively showed that adenylic acid is attached at O-2 of the 3-amino-3-deoxy-D-glucose moiety of 3',4'-dideoxykanamycin B. The enzyme that transfers the adenylyl group to the 2''-hydroxyl group also transfers the guanylyl group and the inosinyl group from guanosine 5'-triphosphate or inosine 5'-triphosphate to the 2''-hydroxyl group of 3',4'-dideoxykanamycin B. These transfers were confirmed by isolation of 3',4'-dideoxykanamycin B 2''-inosinate and 3',4'-dideoxykanamycin B 2''-guanylate.³⁹

As 3',4'-dideoxykanamycin B and the gentamicins are inactivated by the enzyme prepared from *E. coli* K12 JR66/W677, it follows that the inactivated gentamicins C_{1a}, C₁, and C₂ should be their 2''-adenylates. This enzyme also transfers^{20a} the adenylyl group from adenosine 5'-triphosphate to the 2''-hydroxyl group of the kanamycins and 4-O-(6-amino-6-deoxy- α -D-glucopyranosyl)-6-O- α -D-glucopyranosyl-2-deoxy-streptamine.⁴⁰ From the activity described, this enzyme can be called gentamicin-kanamycin nucleotidyl transferase.

The involvement of this enzyme in the mechanism of resistance has been shown by the fact that 2''-deoxygentamicin C₂ (synthesized by Daniels⁴¹) inhibits *Klebsiella pneumoniae*, which produces an enzyme

(39) M. Yagisawa, H. Naganawa, S. Kondo, T. Takeuchi, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 492 (1972).

(40) M. Murase, T. Ito, S. Fukatsu, and H. Umezawa, in "Progress in Antimicrobial and Anticancer Chemotherapy" (Proceedings of the 6th International Congress of Chemotherapy), University of Tokyo Press, Tokyo, 1970, Vol. 2, p. 1098.

(41) P. J. L. Daniels, *Abstr. Papers Amer. Chem. Soc. Meeting*, **165**, MEDI-5 (1973).

adenylylating the 2"-hydroxyl group of this antibiotic. Inspection of a molecular model reveals the close proximity of the 2"-hydroxyl group and the 1-amino group in the gentamicins and the kanamycins. Therefore, it is readily conceivable that modification of the 1-amino group would interfere with the enzyme reaction. As will be described later (see p. 223), kanamycin derivatives that have an L-4-amino-2-hydroxybutyryl group on the 1-amino group inhibit the resistant strains producing this nucleotidyl transferase.

IV. KANAMYCIN-NEOMYCIN ACETYL TRANSFERASE

Among the enzymes involved in the resistance mechanism, kanamycin-neomycin acetyl transferase was first found by Umezawa and coworkers⁵ in *Escherichia coli* K12 R5 carrying R factor. This strain was obtained by transmission of R factor from naturally isolated, drug-resistant, dysentery bacteria.

The supernatant liquor (S-100 solution) from the centrifugation of disrupted cells of this resistant strain at 100,000g was obtained by a procedure similar to that already described for kanamycin-neomycin phosphate transferase I (see p. 186). Kanamycin A was inactivated in a reaction mixture that consisted of the following materials in the following volume ratios: S-100 solution (10 mg of protein per ml), 4 vols.; a ten-times concentrated buffer solution (0.6 M potassium chloride, 0.1 M magnesium acetate, 0.06 M 2-mercaptoethanol, 1.0 M Tris hydrochloride, pH 7.8), 2 vols.; 165 mM adenosine 5'-triphosphate, 1 vol.; 0.08 M creatine phosphate, 0.5 vol.; 1.2 mg/ml of creatine kinase, 0.5 vol.; 1.2 mM coenzyme A, 1 vol.; and the solution⁴² of the antibiotic, 1 vol. The inactivated kanamycin was extracted and purified by chromatography on a column of silicic acid, followed by chromatography on Dowex-1 X-2 (OH⁻) resin.

Refluxing of the inactivated kanamycin A in 2 M sodium hydroxide for 9 hours gave kanamycin A, and under these conditions, no decomposition of kanamycin A occurred; therefore, this method is useful in determination of the inactivated kanamycin. The inactivated kanamycin melted at 176–180° (bubbling). The formula of monoacetylkanamycin A was obtained by elementary analysis. The amide bond was shown by i.r. spectroscopy. Mono-N-acetylkanamycin was shown by the presence of three nitrogen atoms (Van Slyke) and by the behavior in high-voltage electrophoresis, in comparison with mono-, di-, tri-, and tetra-N-acetylkanamycin. The amount of periodate consumed per mole at pH 4.3 in 1 M sodium acetate buffer at 24° was the same as that per mole of kanamycin A. The inactivated kanamycin A was deaminated by the Van Slyke

(42) M. Okanishi, S. Kondo, Y. Suzuki, S. Okamoto, and H. Umezawa, *J. Antibiot.* (Tokyo), **20**, 132 (1967).

method, and hydrolysis of the deaminated product by refluxing in 6 M hydrochloric acid for 30 minutes gave 6-amino-6-deoxy-D-glucose. Thus, it was shown that the enzyme transfers an acetyl group from acetyl-coenzyme A to the 6'-amino group of kanamycin A.

Escherichia coli K12 R5 (which produced this enzyme) was weakly resistant to kanamycins A and B, but sensitive to kanamycin C, which has no 6'-amino group to be acetylated. The involvement of this enzyme in the mechanism of resistance was shown by the resistance of this organism to kanamycin A and 3',4'-dideoxykanamycin B, and by its sensitive behavior to the 6'-N-methyl derivatives of these antibiotics.

A kanamycin-neomycin acetyl transferase probably the same as that just described was extracted by Benveniste and Davies⁴³ from *E. coli* W677 carrying either R factor R5, NR79, or NR79-5. After the reaction of the antibiotics with the enzyme, they extracted N-acetylkanamycin, N-acetylkanamycin B, N-acetylneomycin B, and N-acetylgentamicin C_{1a}. This enzyme was purified approximately 10-fold from an "osmotic shockate" of strain NR79/W677 by precipitation of the nucleic acids, ammonium sulfate fractionation, and column chromatography on DEAE-cellulose. It required magnesium ion both for activity and stability. Acetyl coenzyme A protected the enzyme against inactivation in a buffer at 42°. The optimal pH for acetylation of kanamycin A was 5.8. The position of the acetyl group was not determined, but the substrate specificity suggests that the 6'-amino group is acetylated.

Kanamycin acetyl transferase was also found in *Pseudomonas aeruginosa* GN315, which was resistant to kanamycin, 3',4'-dideoxykanamycin B, neomycins, and ribostamycin.^{44,45}

The enzyme solution was prepared from logarithmically growing cells of *Pseudomonas aeruginosa* GN315 in a nutrient broth containing 5 µg of 3',4'-dideoxykanamycin B per ml. The cells were harvested by centrifugation, and washed twice with buffer A (20 mM potassium phosphate buffer, pH 7.8, containing 6 mM 2-mercaptoethanol). The washed cells were suspended in an equal volume of buffer A, and disrupted by passage through a French pressure-cell (1,200 Mg.cm⁻²). The ruptured-cell suspension was centrifuged at 105,000g for 90 minutes, and the supernatant liquor was dialyzed overnight against buffer A. The dialyzed enzyme solution was diluted with buffer A to 20 mg of protein per ml.

Inactivation was conducted for 3 hours at 37° in the following reaction mixture: 90 mg (0.2 mmole) of 3',4'-dideoxykanamycin B in 25 ml of distilled water, 4.842 g (8.0 mmoles) of disodium adenosine 5'-triphosphate in 100 ml of 0.8% sodium hydrogen carbonate, 50 mg (0.06 mmole) of trisodium coenzyme A in 50 ml of

(43) R. Benveniste and J. Davies, *Biochemistry*, **10**, 178 (1971).

(44) H. Kawabe and S. Mitsuhashi, *Japan. J. Microbiol.*, **16**, 436 (1972).

(45) H. Yamamoto, M. Yagisawa, H. Naganawa, S. Kondo, T. Takeuchi, and H. Umezawa, *J. Antibiot. (Tokyo)*, **25**, 746 (1972).

distilled water, 25 ml of the enzyme solution, 25 ml of 100 mM magnesium acetate–60 mM 2-mercaptoethanol, and 25 ml of 1 M potassium phosphate buffer, pH 7.8. After incubation for 3 hours, 94% of the 3',4'-dideoxykanamycin B was inactivated.

The reaction mixture was diluted with 250 ml of distilled water, and the solution kept in a boiling-water bath for 10 minutes; the suspension was filtered, and the filtrate was passed through a column (15 ml) of Amberlite CG-50 (NH_4^+) resin, which was washed with 2 liters of distilled water, and the inactivated 3',4'-dideoxykanamycin B then eluted with 0.1 M ammonia. The eluate, which gave positive ninhydrin and Rydon–Smith reactions, was evaporated to dryness, yielding 96 mg of a brownish powder. The powder was rechromatographed on a column of Amberlite CG-50 resin, yielding 88 mg of a yellowish powder, which was further subjected to chromatography on CM-Sephadex C-25 equilibrated with 0.1 M ammonium formate, the inactivated 3',4'-dideoxykanamycin B being eluted therefrom with 0.6 M ammonium formate. After chromatography on Amberlite CG-50 resin to remove formic acid, evaporation of the 0.1 M ammonia eluate *in vacuo* yielded 69 mg of purified, inactivated 3',4'-dideoxykanamycin B as a white powder.⁴⁶

The inactivated 3',4'-dideoxykanamycin B melted at 146–149°. The empirical formula of 3',4'-dideoxykanamycin B monoacetate was shown by elementary analysis. It gave positive ninhydrin and Rydon–Smith reactions. On high-voltage paper-electrophoresis at 3.5 kV for 15 minutes, with 1:3:36 (v/v) formic acid–acetic acid–water, the inactivated 3',4'-dideoxykanamycin B moved 11.2 cm towards the cathode, whereas 3',4'-dideoxykanamycin B moved 13.5 cm. The infrared spectrum of the inactivated 3',4'-dideoxykanamycin B showed Amide bands I and II. Hydrolysis of the inactivated 3',4'-dideoxykanamycin B with 2 M sodium hydroxide regenerated 3',4'-dideoxykanamycin B. These results constitute proof that the compound is a mono-*N*-acetyl derivative of 3',4'-dideoxykanamycin B.

The n.m.r. spectrum of 3',4'-dideoxykanamycin B has been studied in detail. In the n.m.r. spectrum of the inactivated 3',4'-dideoxykanamycin B in deuterium oxide, with tetramethylsilane as the external reference standard, one *N*-acetyl signal was seen at δ 2.43. Application of the double-resonance technique showed the signal of the C-6' methylene protons at δ 3.15 for 3',4'-dideoxykanamycin B shifted to δ 3.7 for the inactivated 3',4'-dideoxykanamycin B. Thus, the structure of the inactivated 3',4'-dideoxykanamycin B was determined⁴⁶ to be 6'-*N*-acetyl-3',4'-dideoxykanamycin B.

As described in the next Section, the gentamicins and 3',4'-dideoxykanamycin B show molecular-ion peaks in mass spectrometry, and thus this method is useful in determining the structures of the inactivated derivatives of these antibiotics. This structure was also consistent with

(46) M. Yagisawa, H. Naganawa, S. Kondo, T. Takeuchi, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 495 (1972).

its mass spectrum: a peak of a mono-*N*-acetyl-3',4'-dideoxykanamycin B at m/e 494 ($M + 1$), and an intense peak at m/e 171 attributed⁴⁶ to the *N*-acetyl-3',4'-dideoxyneosome C unit.

By a similar procedure, after the reaction of kanamycin A in the reaction mixture already described, 6'-*N*-acetylkanamycin (kanamycin 6'-acetate), m.p. 162–172°, was isolated.⁴⁶

In a similar way, inactivated ribostamycin was isolated. The inactivated ribostamycin is a white powder melting at 103–108° (dec.). The empirical formula of monoacetylribostamycin monohydrate was obtained by elementary analysis. It shows Amide bands I and II in its i.r. spectrum, and one *N*-acetyl signal (at δ 2.49) in its n.m.r. spectrum. Double-resonance experiments showed that the signal of the methylene protons on C-6' had been shifted to lower field, being at δ 3.5 in ribostamycin, and δ 3.8 in the inactivated ribostamycin, thus indicating that the inactivated ribostamycin is 6'-*N*-acetylribostamycin.⁴⁵ Involvement of this enzyme in the mechanism of resistance can be shown by the fact that *Pseudomonas aeruginosa* GN315 is not inhibited by 100 μ g of 3',4'-dideoxykanamycin B, but is inhibited⁴⁷ by 12.5 μ g of 3',4'-dideoxy-6'-*N*-methylkanamycin B per ml.

V. GENTAMICIN ACETYL TRANSFERASE

Mitsuhashi and coworkers⁴⁸ first reported that the supernatant liquor from centrifugation (at 105,000g) of disrupted cells of *Pseudomonas aeruginosa* 99 and *Pseudomonas aeruginosa* Cape 18 inactivates gentamicins C_{1a}, C₁, and C₂ in the acetylating system described in a previous Section (see p. 209). A similar enzyme in *Pseudomonas aeruginosa* 130 and 209 was studied by Davies and coworkers⁴⁹ more soundly, as they isolated the inactivated gentamicin C_{1a} and determined its structure. As described by these authors, this enzyme catalyzes the transfer of the acetyl group from acetyl coenzyme A to the 3-amino group of the 2-deoxystreptamine moiety of the gentamicins. It is specific to gentamicins, and does not acetylate kanamycins. *Pseudomonas aeruginosa* 130 and 209 were found to be resistant to neomycin B, kanamycins A, B, and C, paromomycin, and the gentamicins, but to be inhibited by 20 μ g of tobramycin (3'-deoxykanamycin B) per ml.

- (47) H. Umezawa, Y. Nishimura, T. Tsuchiya, and S. Umezawa, *J. Antibiot.* (Tokyo), **25**, 743 (1972).
- (48) S. Mitsuhashi, F. Kobayashi, and M. Yamaguchi, *J. Antibiot.* (Tokyo), **24**, 400 (1971).
- (49) M. Brzezinska, R. Benveniste, J. Davies, P. J. L. Daniels, and J. Weinstein, *Biochemistry*, **11**, 761 (1972).

Pseudomonas aeruginosa 130 was grown to the late logarithmic phase in yeast extract-tryptone medium. The cells were harvested by centrifugation, and washed with 10 mM Tris-50 mM ammonium chloride (pH 7.8) at 4°. The pellet (25 g) was resuspended in 130 ml of the same buffer at 4° (from this point, all manipulations were performed at 4–10°), and disrupted by sonication. The cell debris was removed by centrifugation at 30,000g for 30 minutes. The supernatant liquor was centrifuged at 105,000g to remove ribosomes, nucleic acids were precipitated by the addition of streptomycin sulfate to a final concentration of 1.5%, and the resulting supernatant liquor was adjusted to a protein concentration of 10 mg/ml, and made 35% in ammonium sulfate. The precipitate was removed by centrifugation, the supernatant liquor was adjusted to 50% ammonium sulfate, and the precipitate was collected by centrifugation, dissolved in 3 ml of 10 mM Tris buffer (pH 7.8), and the solution dialyzed exhaustively against the same buffer. The precipitate (35–50% ammonium sulfate), after dialysis, was applied to a column (1.5 × 17.5 cm) of DEAE-cellulose pre-equilibrated with 10 mM Tris buffer, pH 7.8. A linear gradient (400 ml) with 0 to 0.3 M ammonium chloride in 10 mM Tris buffer, pH 7.8, was applied at a flow rate of 20 ml/hr. Fractions were assayed for gentamicin-acetylating activity, and the enzyme was found to be eluted at 0.22 M ammonium chloride. The fractions containing the enzyme activity were combined, concentrated by ultrafiltration, and dialyzed against 10 mM Tris buffer, pH 7.8. Thus, the enzyme was purified about 15-fold.

The enzyme was assayed for acetylating activity by use of ^{14}C -acetyl coenzyme A.

The reaction mixture for large-scale acetylation of gentamicin C_{1a} contained, in a total volume of 150 ml, 90 ml of a cell extract obtained by disrupting 30 g of *Pseudomonas aeruginosa* 130 in a French press, an adenosine 5'-triphosphate-generating system consisting of 0.3 mmole of enolpyruvate phosphate and 5 mg of pyruvate kinase, 5 mmoles of adenosine 5'-triphosphate (adjusted to pH 6 with potassium hydroxide), 20 μ moles of yeast coenzyme A, 0.23 mmole of gentamicin C_{1a} , 15 mmoles of Tris hydrochloride (pH 7.6 at 30°), 1.5 mmoles of magnesium acetate, 6 mmoles of ammonium chloride, 6 mmoles of potassium chloride, and 0.75 mmole of 1,4-dithiothreitol. Incubation was performed for 6 hours at 33° with gentle agitation. Sixty percent of the gentamicin C_{1a} was acetylated after 6 hours. The inactivated gentamicin C_{1a} was extracted by chromatography on a cation-exchange resin.

Acetylgentamicin C_{1a} is an amorphous material, m.p. 130–145°, $[\alpha]_D^{26} +126^\circ$ (c 0.37, water). Hydrolysis with 2 M sodium hydroxide regenerated a substantial proportion of gentamicin C_{1a} . The compound shows Amide bands I and II in its i.r. spectrum. The n.m.r. spectrum shows a single acetyl-group signal at δ 1.96. As reported by Daniels and co-workers,⁵⁰ mass-spectral analysis is useful for gentamicins which give a molecular-ion peak. The mass spectrum of the inactivated gentamicin C_{1a} exhibits a molecular-ion peak at m/e 491, and a somewhat more

(50) P. J. L. Daniels, M. Kugelman, A. K. Mallams, R. W. Tkach, H. F. Vernay, J. Weinstein, and A. Yehaskel, *J. Chem. Soc. (D)*, 1629 (1971).

intense $(M + 1)^+$ peak at m/e 492 consistent with a mono-*N*-acetylated gentamicin C_{1a} . Intense peaks at m/e 160 and 129, attributed to the garosamine and purpurosamine C_{1a} ions, indicate that the acetyl group is not attached to either of these parts of the molecule. Instead of the peaks at m/e 191, 163, and 145 associated with the 2-deoxystreptamine unit in gentamicin C_{1a} , a series of peaks 42 mass units higher, at m/e 233, 205, and 187, are present in the spectrum of inactivated gentamicin C_{1a} , indicating that the *N*-acetyl group is attached to one of the nitrogen atoms of 2-deoxystreptamine. Methanolysis of the *N*-acetylated gentamicin C_{1a} afforded the corresponding *N*-acetylgentamine C_{1a} as an amorphous material; $[\alpha]_D^{26} + 50^\circ$ (c 0.18, water); n.m.r. data: δ 2.01 p.p.m. (3 H, singlet, acetyl methyl group). The presence of an acetyl group on the 3-amino group of the 2-deoxystreptamine moiety was determined by examining the circular dichroism in "cupra A" and tetraamminecopper sulfate solutions.

The enzyme solution prepared from *Pseudomonas aeruginosa* 99 by Mitsuhashi and coworkers⁵¹ required magnesium ion, and had a substrate specificity similar to that of the enzyme solution prepared from *Pseudomonas aeruginosa* 130 as just described. The optimal pH was 6.5. These authors⁵¹ isolated the inactivated product of gentamicin C_1 , and mass spectrometry thereof indicated an *N*-acetyl group present in the 2-deoxystreptamine moiety. The placement as a 3-*N*-acetyl group was shown by periodate oxidation of a methanolysis product, namely, *N*-acetylgentamine, which consumes 2 moles of periodate per mole.

The purification of gentamicin acetyl transferase can also be achieved by affinity chromatography, with a gradient of sodium chloride from 0.5 to 2 *M*, by using Indubiose 4A-gentamicin C_1 or Indubiose 4A-kanamycin, which were prepared by mixing cyanogen bromide-activated Indubiose 4A with these antibiotics.⁵² Involvement of this enzyme in the mechanism of resistance to gentamicins is certain, because *Pseudomonas aeruginosa* 99 is inhibited by 3',4'-dideoxykanamycin B.

Witchitz⁵³ found *Enterobacter*, *Serratia*, *Klebsiella*, and *Escherichia* species from which R factor causing resistance to gentamicin could be transferred into *E. coli* K12. *E. coli* K12 R135, R136, R137, R138, R140, R141, and R146 thus prepared were found to be resistant to gentamicin C, and sensitive to tobramycin (3'-deoxykanamycin B), kanamycin, paromomycin, and lividomycin. The enzymes produced by one of these

(51) F. Kobayashi, M. Yamaguchi, J. Eda, M. Hiramatsu, and S. Mitsuhashi, *Gunma J. Med. Sci.*, **5**, 291 (1972).

(52) F. LeGoffic and N. Moreau, *FEBS Lett.*, **29**, 289 (1973).

(53) J. L. Witchitz, *J. Antibiot. (Tokyo)*, **25**, 622 (1972).

strains was studied by Umezawa and coworkers,⁵⁴ who confirmed that this strain produces gentamicin acetyl transferase, which acetylates the 3-amino group of this antibiotic.

The enzyme solution was prepared from logarithmically growing cells of the strain in a nutrient broth containing 10 μ g of gentamicin C sulfate per ml. The cells were harvested by centrifugation, and washed twice with 20 mM potassium phosphate buffer (pH 7.2) containing 10 mM magnesium acetate, 60 mM potassium chloride, and 10 mM 2-mercaptoethanol. The washed cells were suspended in an equal volume of the buffer, and disrupted by passage through a French pressure cell (1.2 Mg.cm⁻²). The suspension of ruptured cells was centrifuged at 100,000g for 90 minutes, and the supernatant liquor was diluted with the buffer to 10 mg of protein per ml.

The inactivation of gentamicin C₁ (95.5 mg, 200 μ moles) was achieved in a reaction mixture (200 ml) containing 50 ml of S-100, 2.421 g (4.0 mmoles) of disodium adenosine 5'-triphosphate, 25 mg (30 μ moles) of coenzyme A, 20 ml of 100 mM magnesium acetate, 20 ml of 600 mM potassium chloride, 20 ml of 100 mM 2-mercaptoethanol, and 20 ml of 1 M potassium phosphate buffer (pH 7.2). After incubation for 3 hours at 37°, the antibiotic activity had been completely lost.

The reaction mixture was diluted with 200 ml of water, the solution kept in a boiling-water bath for 10 minutes, filtered, and the filtrate passed through a column (50 ml) of Amberlite CG-50 (NH₄⁺) ion-exchange resin. The column was washed with 2 liters of water, and the inactivated gentamicin C₁ was then eluted with 0.1 M ammonia. The eluate, which was positive in the ninhydrin and Rydon-Smith reactions, was evaporated to dryness, yielding 101 mg of a yellowish powder; this was subjected to chromatography on a column (20 ml) of Amberlite CG-50 (NH₄⁺) resin. The purified, inactivated gentamicin C₁ was eluted with 0.05 M ammonia, and obtained as a white powder (86 mg), m.p. 96–104°.

In the n.m.r. spectrum of the inactivated gentamicin C₁ in deuterium oxide, with tetramethylsilane as the external reference standard, one *N*-acetyl signal was observed (at δ 2.45). The signals of H-2' and H-3'' of both gentamicin C₁ and the inactivated material were shown by the INDOR method to fall in the same region (δ 3.3–3.5), indicating absence of acetylation on NH₂-2' and NH₂-3''. The high-resolution mass-spectrum gave the following signals: *m/e* 520.3331 (6%; Calc. for C₂₃H₄₆N₅O₈: 520.3343, *M* + 1), 233.1172 (21%; Calc. for C₉H₁₇N₂O₅: 233.1136, the first fragment from the *N*-acetyl-2-deoxystreptamine moiety), 215.1032 (15%; Calc. for C₉H₁₅N₂O₄: 215.1031, the second fragment from *N*-acetyl-2-deoxystreptamine), 205.1196 (23%; Calc. for C₈H₁₇N₂O₄: 205.1188, the third fragment from *N*-acetyl-2-deoxystreptamine), 187.1075 (21%; Calc. for C₈H₁₅N₂O₃: 187.1081, the fourth fragment from *N*-acetyl-2-deoxystreptamine), 160.0993 (66%; Calc. for C₇H₁₄NO₃: 160.0973, from garosamine), and 157.1355 (100%; Calc. for C₈H₁₇N₂O: 157.1340, from pur-

(54) H. Umezawa, M. Yagisawa, Y. Matsushashi, H. Naganawa, H. Yamamoto, S. Kondo, T. Takeuchi, Y. A. Chabbert, *J. Antibiot.* (Tokyo), **26**, 612 (1973).

purosamine). This fragmentation was in good agreement with the fragmentation pattern of aminocyclitol antibiotics reported by Daniels and coworkers,⁵⁰ and indicated that one of the amino groups of the 2-deoxystreptamine moiety is acetylated.

From the hydrolyzate obtained by treatment of the tetra-*N*-(ethoxycarbonyl) derivative of the inactivated gentamicin C₁ with 6 *M* hydrochloric acid for 30 minutes at 100°, levorotatory 1-*N*-(ethoxycarbonyl)-2-deoxystreptamine, the antipode of 3-*N*-(ethoxycarbonyl)-2-deoxystreptamine, was isolated by resin chromatography. Consequently, the structure of the inactivated gentamicin C₁ was confirmed to be 3-*N*-acetylgentamicin C₁. It was confirmed⁵⁴ that the enzyme solution acetylates gentamicin C_{1a} and C₁, but not kanamycin and 3',4'-dideoxykanamycin B.

The involvement of this enzyme in the mechanism of resistance of *E. coli* carrying R factor is certain, because the *Escherichia* are inhibited by kanamycins A and B, and 3',4'-dideoxykanamycin B.

VI. STREPTOMYCIN ADENYLYL TRANSFERASE

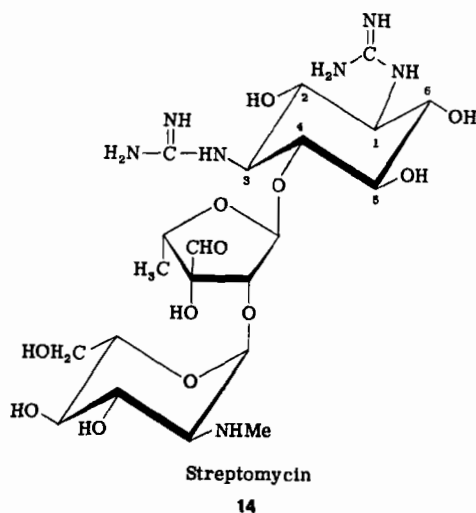
Umezawa and coworkers⁵⁵ first described streptomycin adenylyl transferase in *E. coli* K12 ML1629 carrying an R factor resistant to streptomycin, kanamycins A, B, and C, paromamine, neamine, paromomycin, neomycin, chloramphenicol, and tetracycline, and confirmed that the inactivated product is adenylylstreptomycin.

The supernatant liquor from the disrupted cells centrifuged at 100,000*g* was treated with 2 μ g of deoxyribonuclease per ml and 2 μ g of crystalline ribonuclease per ml at 37° for 15 minutes, and then dialyzed against a buffer solution (0.06 *M* potassium chloride, 0.01 *M* magnesium chloride, and 6 mM 2-mercaptoethanol in 0.1 *M* Tris buffer, pH 7.9). The enzyme solution thus prepared was diluted with the buffer so as to contain 10 mg of protein per ml. Streptomycin sulfate (560 mg) was inactivated in 100 ml of a reaction mixture containing 3.600 g of disodium adenosine 5'-triphosphate, 980 mg of sodium hydrogen carbonate, 40 ml of 10-times concentrated buffer solution, and 30 ml of the enzyme solution. After 20 hours at 37°, 80% of the streptomycin had been inactivated. After the reaction, the reaction mixture was passed through a column (200 ml) of Amberlite IRC-50 (Na⁺) resin, and the inactivated streptomycin on the column was eluted with 0.5 *M* hydrochloric acid. The fraction that gave a positive Sakaguchi reaction and that showed no antibacterial activity was collected and passed through a column (20 g) of active carbon. The inactivated streptomycin was eluted with 1:1 0.2 *M* hydrochloric acid-methanol. The fraction giving a positive Sakaguchi reaction and showing no biological activity was selected, and made neutral with Dowex 44 (OH⁻) resin. The solution was concentrated under vacuum, the inactivated streptomycin (250 mg) was precipitated by addition of 14 volumes of acetone, and the precipitate dried *in vacuo*. It showed

(55) H. Umezawa, S. Takasawa, M. Okanishi, and R. Utahara, *J. Antibiot.* (Tokyo), **21**, 81 (1968).

one spot with the following paper-chromatographic systems: R_F 0.18 (water adjusted to pH 10.0 with aqueous ammonia), R_F 0.08 (50:25:6:19 methanol-ethanol-conc. hydrochloric acid-water).

In high-voltage paper-electrophoresis with 3:1:36 acetic acid-formic acid-water under 3.50 kV for 15 minutes, the inactivated streptomycin moved 10.5 cm towards the cathode and streptomycin moved 13.0 cm, indicating that the inactivated streptomycin is less basic than streptomycin. It gave a positive reaction with the Hanes reagent. A solution of the inactivated streptomycin in distilled water showed a u.v. maximum at 260 nm. Determination of streptomycin by the maltol reaction (by use of the optical absorbance at 550 nm) indicated the presence of streptomycin and adenylic acid in the equimolar ratio of 1:1. The empirical formula of monoadenylylstreptomycin dihydrochloride tetrahydrate was shown by elementary analysis. The inactivated streptomycin was hydrolyzed by phosphate diesterase⁵⁵ to streptomycin (14) and 5'-adenylic acid.



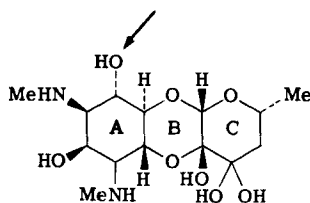
The complete structure of the inactivated streptomycin was determined by Umezawa and coworkers⁵⁶ and Davies and coworkers.⁵⁷ Methanolysis of the inactivated streptomycin in 0.5 M hydrogen chloride in methanol for 24 hours at 30° gave methyl adenylylstreptobiosaminide dimethyl

(56) S. Takasawa, R. Utahara, M. Okanishi, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **21**, 477 (1968).

(57) T. Yamada, D. Tipper, and J. Davies, *Nature*, **219**, 288 (1968).

acetal, which consumed one mole of periodate per mole, whereas methyl streptobiosaminide dimethyl acetal consumes 2 moles per mole. Ethanol extraction of the acid hydrolyzate of the periodate-oxidation products of the inactivated streptomycin and of methyl adenylylstreptobiosaminide dimethyl acetal gave 2-deoxy-2-(methylamino)-L-glucose, identified by comparison with an authentic sample obtained by hydrolysis of dihydrostreptomycin in 0.5 M sulfuric acid for 50 hours at 50°. Based on these data, Umezawa and coworkers⁵⁶ proposed the structure of 3''-O-adenylylstreptomycin for the inactivated streptomycin. Davies and coworkers⁵⁷ extracted the enzyme from *E. coli* JE254 by osmotic shock, and detected 2-deoxy-2-(methylamino)-L-glucose after hydrolysis of the product from periodate oxidation of the inactivated streptomycin. The enzyme obtained from both strains required magnesium ion for the reaction, and the optimal pH was 8.3–8.5.

Davies and coworkers⁵⁸ found that streptomycin adenylyl transferase also adenylylates spectinomycin and actinamine. Because of the stereochemical resemblance between actinamine and the *D-threo* methylamino alcohol moiety in streptomycin, the site of adenylylation was considered to be that shown by the arrow in formula 15.



15

Biochemical and genetic studies by D. H. Smith and coworkers⁵⁹ indicated that adenylylation of streptomycin, dihydrostreptomycin, bluensomycin, actinamine, spectinomycin, and dihydrospectinomycin is catalyzed by a single enzyme.

Kawabe and Mitsuhashi⁶⁰ reported that the streptomycin-inactivating enzyme in staphylococci of medium resistance seems to be of the streptomycin adenylyl transferase type, because the ¹⁴C-adenosine residue

(58) R. Benveniste, T. Yamada, and J. Davies, *Infec. Immunity*, **1**, 109 (1970).

(59) D. H. Smith, J. N. Prescott, and P. W. Anderson, *Infec. Immunity*, **1**, 120 (1970).

(60) H. Kawabe and S. Mitsuhashi, *Japan. J. Microbiol.*, **15**, 545 (1971).

in ^{14}C -adenosine 5'-triphosphate is incorporated into the inactivated dihydrostreptomycin.

S. Umezawa and coworkers have now achieved a total synthesis of dihydrostreptomycin, and derivatives resistant to adenylation or phosphorylation (described in the next Section) and which inhibit resistant organisms will presumably be synthesized. In this connection, a paper⁶¹ on the X-ray analysis of streptomycin oxime may be mentioned.

VII. STREPTOMYCIN PHOSPHATE TRANSFERASE

As already described, *Escherichia coli* carrying R factor which produces streptomycin adenylyl transferase is resistant to spectinomycin. Davies and coworkers⁶² found R factor strains that were resistant to streptomycin but not to spectinomycin. The enzyme was prepared from one such strain, *E. coli* JR35, by osmotic shock, and extracted by ammonium sulfate precipitation followed by chromatography on DEAE-cellulose, Biogel P-30, and hydroxylapatite. The transphosphorylation from adenosine 5'-triphosphate to streptomycin by this enzyme required magnesium ion (which could be replaced by zinc ion). It also phosphorylated methyl streptobiosaminide. Spectinomycin did not undergo this enzyme reaction. The inactivated streptomycin was converted into streptomycin by alkaline phosphatase. Hydrolysis of the periodate-oxidation product gave 2-deoxy-2-(methylamino)-L-glucose, and the 3''-hydroxyl group was shown to be phosphorylated.

Mitsubishi and coworkers⁶³ found streptomycin phosphate transferase in *Pseudomonas aeruginosa* TI-13; they isolated the inactivated compound, which was shown to have the empirical formula of a mono-O-phosphonodihydrostreptomycin monocarbonate trihydrate by elementary analysis. They also found⁶⁴ the same enzyme in other strains, namely, 99, 137, 138, 351, 10126, Cape 18, and TI-11. The enzyme in the supernatant liquor (600 ml) from centrifugation of disrupted cells of strain TI-13 at 105,000g was precipitated by ammonium sulfate at 33–66% saturation. Further purification by successive, column chromatography on Sephadex G-75, DEAE-Sephadex A-50 (with a gradient of potassium

(61) S. Neidle, D. Rogers, and M. B. Hursthouse, *Tetrahedron Lett.*, 4725 (1968).

(62) B. Ozanne, R. Benveniste, D. Tipper, and J. Davies, *J. Bacteriol.*, **100**, 1144 (1969).

(63) F. Kobayashi, M. Yamaguchi, and S. Mitsunashi, *Japan. J. Microbiol.*, **15**, 381 (1971).

(64) F. Kobayashi, M. Yamaguchi, J. Sato, and S. Mitsunashi, *Japan. J. Microbiol.*, **16**, 15 (1972).

chloride from 0.1 to 0.5 *M* in a buffer), Sephadex G-75, and DEAE-Sephadex gave 205-fold purification, in a recovery of 2.9%. Magnesium ion was required for the reaction. The optimal pH of the enzyme activity was ~ 10.0 . The activity of the enzyme was not decreased when it was heated for 5 minutes at 50°, but was completely lost when it was heated for 5 minutes at 70°.

Naganawa and coworkers¹⁵ described the determination of the structure of dihydrostreptomycin 3''-phosphate by application of n.m.r. spectroscopy with the double-resonance technique. The n.m.r. spectra of dihydrostreptomycin sulfate and dihydrostreptomycin 3''-phosphate sulfate both showed two signals in the anomeric regions. Irradiation at δ 5.78 (H-1') collapsed the doublet at δ 4.75 (H-2', $J_{1',2'}$ 1.6 Hz) to a sharp singlet. Irradiation of the signal at the center of δ 4.76 (H-4', $J_{4',5'}$ 6.5 Hz, q) overlapping with the signal at δ 4.75 (H-2') produced a singlet at δ 1.68 ($J_{4',5'}$ 6.5 Hz, d) for the methyl group of dihydrostreptose, and the doublet of the anomeric proton of dihydrostreptose collapsed to a singlet at δ 5.78. Thus, the signal for the anomeric proton of the 2-deoxy-2-(methylamino)- α -L-glucopyranoside moiety should be the signal at δ 6.01 (H-1'', J 3.5 Hz, d). Irradiation of the signal at δ 6.01 collapsed the doublet of doublets signal at δ 3.77 (H-2'', J 10.5, 3.6 Hz) to a doublet (J 10.5 Hz). Irradiation at δ 3.77 collapsed the doublet of doublets signal at δ 4.43 (H-3'', J 10.5, 8.0 Hz) to a doublet (H-3'', J 8.0 Hz), and a doublet signal at δ 6.01 (J 3.5 Hz) collapsed to a singlet (H-1''). Accordingly, the signals at δ 6.01, 3.77, and 4.43 can be assigned to H-1'', H-2'', and H-3'', respectively, of 2-deoxy-2-(methylamino)-L-glucose. For dihydrostreptomycin 3''-phosphate, the H-3'' signal was observed at $\delta \sim 4.8$ overlapping with the H-2' and H-4' signals for streptose. Irradiation at δ 4.8 collapsed the doublet of doublets signal at δ 3.94 (J 10.3, 3.0 Hz, H-2''), the doublet signal at δ 5.74 (J 1.6 Hz, H-1'), and the doublet signal at δ 1.68 (J 6.5 Hz, CH₃—C) to a doublet of J 10.3 Hz, a singlet, and a singlet of the methyl group, respectively. Although the splitting pattern of H-3'' in dihydrostreptomycin 3''-phosphate was not observed, the H-3'' signal is shifted to lower field by ~ 0.4 p.p.m. compared with dihydrostreptomycin. These results indicated that the phosphate group is attached at C-3 in the 2-deoxy-2-(methylamino)-L-glucose residue.

VIII. DEVELOPMENT OF ACTIVE DERIVATIVES

Aminoglycosidic antibiotics inhibit protein synthesis on bacterial ribosomes, but the primary sites that are subject to the action of each of these antibiotics are different and are now under study. As yet, there is no information about the chemical structure of the primary sites, and

information concerning the structure-activity relationship is very slight. However, based on the biochemical mechanism of resistance already described, we can predict the structures of derivatives that will not undergo the reaction of enzymes which are involved in the mechanism of resistance. In this connection, the process of the enzyme reaction has been studied in most detail on kanamycin-neomycin phosphate transferase of *Pseudomonas aeruginosa*. Moreover, as regards substrate structures and inhibitor structures, the other kanamycin-neomycin phosphate transferases are similar to this enzyme. For instance, in all cases, the 3-amino-3-deoxy-D-glucose moiety of kanamycins and the D-ribose moiety of ribostamycin are not involved in interaction of these antibiotics with these enzymes. Therefore, the data obtained on kanamycin-neomycin phosphate transferase of *Pseudomonas aeruginosa* may be utilized to predict structures which will inhibit the resistant strains producing these phosphate transferases. Based on the structural moiety involved in binding with the enzyme, or based on the hydroxyl group or the amino group that undergoes the reaction of the enzyme which is the mechanism of resistance, the structures of derivatives not attacked by the enzyme may be predicted. Among such derivatives, active and useful ones may be found. This type of work has been initiated and continued by H. Umezawa with the collaboration of S. Umezawa, who has described the chemical synthesis of such derivatives in Chapter 3 of this Volume. The antibacterial activities of such derivatives against resistant strains are given in Tables III to VII.

As already described, kanamycin-neomycin phosphate transferases which phosphorylate the 3'-hydroxyl group of kanamycins, neomycins, paromomycins, ribostamycin, paromamine, and neamine constitute the mechanism of resistance of most strains of resistant, Gram-negative bacteria, and resistant staphylococci and *Pseudomonas aeruginosa*. Rationalizing from this enzymic mechanism of resistance, 3'-O-methyl derivatives and 3'-deoxy and 3',4'-dideoxy derivatives have been synthesized. 3'-O-Methylkanamycin A (Ref. 65) and 3'-O-methylneamine (Ref. 66) showed no antibacterial activity, but the 3'-deoxy derivatives [3'-deoxykanamycin A (Ref. 8), 3'-deoxykanamycin B (Ref. 67), and 3'-deoxyribostamycin] and the 3',4'-dideoxy derivatives [3',4'-dideoxykanamycin B (Ref. 9), 3',4'-dideoxy-6'-N-methylkanamycin B (Ref. 47),

(65) H. Umezawa, T. Tsuchiya, R. Muto, and S. Umezawa, *Bull. Chem. Soc. Jap.*, **45**, 2842 (1972).

(66) S. Umezawa, T. Jikihara, T. Tsuchiya, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 322 (1972).

(67) Y. Takagi, T. Miyake, T. Tsuchiya, and S. Umezawa, *J. Antibiot.* (Tokyo), **26**, 403 (1973).

TABLE III

Minimum Inhibitory Concentrations ($\mu\text{g/ml}$) of 3'-Deoxykanamycin B (DEKB), 3',4'-Dideoxykanamycin B (DKB), 3',4'-Dideoxy-6'-N-methylkanamycin B (DMKB), and Gentamicins C₁ and C_{1a}

Organism	DEKB	DKB	DMKB	C ₁	C _{1a}
<i>Escherichia coli</i>					
K12	0.78	0.78	1.56	0.39	0.39
R5 ^a	6.25	3.12	1.56	0.39	0.78
ML1629 ^b	0.78	0.78	3.12	1.56	1.56
ML1410 R81 ^b	1.56	1.56	3.12	1.56	0.78
LA290 R55 ^c	25	50	25	100	50
JR66/W677 ^d	50	50	25	50	50
<i>Pseudomonas aeruginosa</i>					
TI-13 ^e	1.56	1.56	6.25	12.5	1.56
GN315 ^e	100	100	12.5	12.5	100
99 ^f	3.12	3.12	6.25	100	>100

^a Kanamycin-neomycin acetyl transferase. ^b Kanamycin-neomycin phosphate transferase I. ^c Gentamicin-kanamycin nucleotidyl transferase. ^d Gentamicin-kanamycin nucleotidyl transferase + kanamycin-neomycin phosphate transferase II. ^e Kanamycin-neomycin phosphate transferase (type II) + phosphate transferase of lividomycin. ^f Gentamicin acetyl transferase.

TABLE IV

Minimum Inhibitory Concentrations ($\mu\text{g/ml}$) of Ribostamycin (RB), 3'-Deoxyribostamycin (DERB), 3',4'-Dideoxyribostamycin (DRB), Butirosin B (BB), and 3',4'-Dideoxybutirosin B (DBB)

Organism*	RB	DERB	DRB	BB	DBB
<i>Escherichia coli</i>					
K12	3.12	0.78	3.12	0.78	1.56
R5 ^a	50	100	100	6.25	6.25
ML1629 ^b	>100	100	>100	1.56	0.78
ML1410 R81 ^b	>100	>100	>100	1.56	0.78
LA290 R55 ^c	3.12	1.56	3.12	0.78	1.56
JR66/W677 ^d	>100	3.12	6.25	>100	3.12
<i>Pseudomonas aeruginosa</i>					
TI-13 ^e	>100	6.25	25	25	12.5
GN315 ^e	>100	>100	>100	>100	>100
99 ^f	>100	6.25	50	50	25

* For key to superscripts, see footnotes to Table III.

TABLE V

Minimum Inhibitory Concentrations ($\mu\text{g/ml}$) of 1-N-(1,4-Amino-2-hydroxybutyryl)neamine (AHB-NA), 3',4'-Dideoxynamine (DNA), and Neamine (NA)

Organism*	AHB-NA	DNA	NA
<i>Escherichia coli</i>			
K12	3.12	6.25	6.25
R5 ^a	50	50	>100
ML1629 ^b	3.12	12.5	>100
ML1410 R81 ^b	12.5	25	>100
LA290 R55 ^c	3.12	6.25	6.25
JR66/W677 ^d	12.5	25	>100
<i>Pseudomonas aeruginosa</i>			
TI-13 ^e	6.25	25	>100
GN315 ^a	>100	>100	>100
99 ^f	25	50	>100

* For key to superscripts, see footnotes to Table III.

3',4'-dideoxyribostamycin (Ref. 20), 3',4'-dideoxybutirosin B (Ref. 23), and 3',4'-dideoxynamine (Ref. 24)] do show antibacterial activity, as seen in Tables III to V.

Lividomycins lack a 3'-hydroxyl group that could undergo the reaction of a kanamycin-neomycin phosphate transferase, but the 5''-hydroxyl group is phosphorylated by kanamycin-neomycin phosphate transferase

TABLE VI

Minimum Inhibitory Concentrations ($\mu\text{g/ml}$) of 1-N-(1,4-Amino-2-hydroxybutyryl) Derivatives (AHB-KM, AHB-KMB, and AHB-DKB) of Kanamycin A, Kanamycin B, and 3',4'-Dideoxykanamycin B (DKB)

Organism*	AHB-KM	AHB-KMB	AHB-DKB	DKB
<i>Escherichia coli</i>				
K12	0.78	0.78	0.78	0.78
R5 ^a	0.39	0.78	0.78	3.12
ML1629 ^b	0.78	1.56	0.78	0.78
ML1410 R81 ^b	1.56	1.56	1.56	1.56
LA290 R55 ^c	0.78	1.56	0.78	50
JR66/W677 ^d	1.56	3.12	1.56	50
<i>Pseudomonas aeruginosa</i>				
TI-13 ^e	3.12	6.25	3.12	1.56
GN315 ^a	100	50	25	>100
99 ^f	6.25	25	12.5	3.12

* For key to superscripts, see footnotes to Table III.

TABLE VII

Minimum Inhibitory Concentrations ($\mu\text{g/ml}$) of Lividomycin A (LMA), 3'-Deoxyneomycin B (DNB), and 1-*N*-(L-4-Amino-2-hydroxybutyryl)-lividomycin A (AHB-LMA)

Organism*	LMA	DNB	AHB-LMA
<i>Escherichia coli</i>			
K12	1.56	0.78	3.12
R5 ^a	0.78	0.39	1.56
ML1629 ^b	>100	50	3.12
ML1410 R81 ^b	>100	100	6.25
LA290 R55 ^c	3.12	1.56	3.12
JR66/W677 ^d	6.25	1.56	6.25
<i>Pseudomonas aeruginosa</i>			
TI-13 ^e	25	0.78	25
GN315 ^e	50	25	50
99 ^f	50	1.56	50

* For key to superscripts, see footnotes to Table III.

I. Occurrence of cross-resistance in high frequency between kanamycin A and lividomycin A in resistant, Gram-negative bacteria and resistant staphylococci is thought to be due to this enzyme. Besides this enzyme, the data described in a previous Section (see p. 203) suggest the presence in *Pseudomonas aeruginosa* of another enzyme which phosphorylates the 5''-hydroxyl group of lividomycins. In the case of lividomycins, 5''-deoxylividomycins A (Ref. 68) and B (Ref. 69) and 5''-amino-5''-deoxylividomycin A (Ref. 68) showed only very weak activity. This result suggests that the 5''-hydroxyl group of the lividomycins may be involved in their action on protein synthesis on the bacterial ribosomes.

The effective group is often found in natural compounds. Butirosin B (Ref. 21) is 1-*N*-(L-4-amino-2-hydroxybutyryl)ribostamycin. This antibiotic inhibits *Escherichia coli* carrying R factor which produces kanamycin-neomycin phosphate transferase I. Therefore, it is possible that the L-4-amino-2-hydroxybutyryl group inhibits binding of the 1-amino group with the enzyme, or causes steric hindrance to the binding of the ribostamycin moiety with the enzyme. As expected, 1-*N*-4-amino-2-hydroxybutyryl derivatives of kanamycin A (BB-K8) (Ref. 70), kana-

- (68) H. Yamamoto, S. Kondo, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 487 (1972).
- (69) S. Umezawa, I. Watanabe, T. Tsuchiya, H. Umezawa, and M. Hamada, *J. Antibiot.* (Tokyo), **25**, 617 (1972).
- (70) H. Kawaguchi, T. Naito, S. Nakagawa, and K. Fujisawa, *J. Antibiot.* (Tokyo), **25**, 695 (1972).

mycin B (Ref. 71), 3',4'-dideoxykanamycin B (Ref. 71), 3',4'-dideoxy-neamine,⁷² and lividomycin A (Ref. 73), as may be seen in Tables V, VI, and VII, inhibit the organisms that produce kanamycin phosphate transferase I. Kanamycin phosphate transferase II also phosphorylates the 3'-hydroxyl group of the butirosins. 1-*N*-(L-4-Amino-2-hydroxybutyryl) derivatives of kanamycins and lividomycin A inhibit *E. coli* JR66/W677, which produces the phosphate transferase II.

From inspection of a molecular model of kanamycins and their derivatives, the 2''-hydroxyl group is seen to be located near to the 1-amino group. Therefore, it may readily be expected that the L-4-amino-2-hydroxybutyryl group on the 1-amino group of the 2-deoxystreptamine moiety would interfere with the reaction of gentamicin-kanamycin nucleotidyl transferase, which adenylylates the 2''-hydroxyl group of these antibiotics. Actually, as may be seen in the Tables, derivatives of this type inhibit the resistant strains which produce this enzyme. Removal of the 2''-deoxy group from gentamicins also gives active derivatives against resistant strains of this type.⁴¹

Escherichia coli R5 has a kanamycin-neomycin acetyl transferase that acetylates the 6'-amino group. However, resistance of this strain to kanamycins is very weak. *Pseudomonas aeruginosa* GN315 is highly resistant to these antibiotics; here, 6'-*N*-methylation of 3',4'-dideoxykanamycin B (Ref. 47) gives a compound that is active against this strain, as may be seen in Table III.

Isolation of resistant strains from patients, and further study of the details of the mechanism of resistance, will provide a fundamental basis for the preparation of active agents useful in the treatment of future resistant infections.

IX. INVOLVEMENT OF ADENOSINE CYCLIC 3',5'-PHOSPHATE IN ENZYME BIOSYNTHESIS

Adenosine cyclic 3',5'-phosphate controls a number of functions in *Escherichia coli*, and its role in regulating the synthesis of inducible enzymes has been studied in detail. Harwood and D. H. Smith⁷⁴ found that adenosine cyclic 3',5'-phosphate regulates the levels of the chlor-

- (71) S. Kondo, K. Iinuma, H. Yamamoto, K. Maeda, and H. Umezawa, *J. Antibiot.* (Tokyo), **26**, 412 (1973).
- (72) S. Umezawa, D. Ikeda, T. Tsuchiya, and H. Umezawa, *J. Antibiot.* (Tokyo), **26**, 304 (1973).
- (73) I. Watanabe, T. Tsuchiya, S. Umezawa, and H. Umezawa, *J. Antibiot.* (Tokyo), **26**, 310 (1973).
- (74) J. Harwood and D. H. Smith, *Biochem. Biophys. Res. Commun.*, **42**, 57 (1971).

amphenicol acetyl transferase and the streptomycin adenylyl transferase. Stimulation of synthesis of kanamycin-neomycin phosphate transferase I by adenosine cyclic 3',5'-phosphate was also observed by Umezawa and coworkers.⁷⁵ These enzymes are not inducible, but are synthesized constitutively. Crombrugghe and coworkers⁷⁶ reported that synthesis of chloramphenicol acetyl transferase is less dependent on adenosine cyclic 3',5'-phosphate receptor protein and adenosine cyclic 3',5'-phosphate than β -D-galactosidase synthesis (which is inducible). In a reaction mixture containing (a) 2'-deoxyribonucleic acid from thermoinducible phage P1CM carrying the gene for chloramphenicol acetyl transferase, (b) a cell-free *E. coli* extract, and (c) the substrates and cofactors needed for synthesis of ribonucleic acid and protein, adenosine cyclic 3',5'-phosphate stimulated the synthesis of chloramphenicol acetyl transferase about fifteen-fold when added to this cell-free system. A clearly measurable synthesis of this enzyme occurred in the absence of adenosine cyclic 3',5'-phosphate. There was an increase of synthesis of this enzyme when guanosine 5'-tetraphosphate was added together with adenosine cyclic 3',5'-phosphate. The stimulation of synthesis of chloramphenicol acetyl transferase by guanosine 5'-tetraphosphate differs from that of inducible enzymes, in that the presence of adenosine cyclic 3',5'-phosphate is not required. Thus, these authors⁷⁶ suggested the possible presence of some unique, structural features at the regulatory regions at the beginning of the operons in the synthesis of R-factor-directed enzymes. Dottin and coworkers⁷⁷ also observed the stimulation of chloroamphenicol acetyl transferase synthesis by adenosine cyclic 3',5'-phosphate *in vitro*, and they suggested the presence of unknown inducers of this enzyme.

Information on the regulation of R-factor-directed synthesis of enzymes involved in the mechanism of resistance will give useful information on the behavior of genetic materials of these enzymes, and may give some clues for finding a new method to prevent the development of resistant cultures.

- (75) I. Tsukada, M. Yagisawa, M. Umezawa, M. Hori, and H. Umezawa, *J. Antibiot.* (Tokyo), **25**, 144 (1972).
- (76) B. Crombrugghe, I. Pastin, W. V. Shaw, and J. L. Rosner, *Nature, New Biology*, **241**, 237 (1973).
- (77) R. P. Dottin, L. S. Shiner, and D. I. Hoar, *Virology*, **51**, 509 (1973).

THE METABOLISM OF α,α -TREHALOSE*

BY ALAN D. ELBEIN

*Department of Biochemistry, The University of Texas Health Science Center,
San Antonio, Texas 78284*

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I. INTRODUCTION

Trehalose is the general name used for the D-glucosyl D-glucosides, of which three isomers containing the pyranose forms of the sugar moieties are known. In general, α,α -trehalose (α -D-glucopyranosyl α -D-glucopyranoside) is the commonly occurring isomer which is widespread in Nature. Thus, this sugar has been isolated from algae, bacteria, fungi, insects, invertebrates, and yeasts, as well as from some lower vascular plants and a few flowering plants. Even in mammalian systems, some of the enzymes involved in the synthesis and degradation of α,α -trehalose are present, although this sugar has not been isolated from these sources. Most of this Chapter will deal with the occurrence and metabolism of

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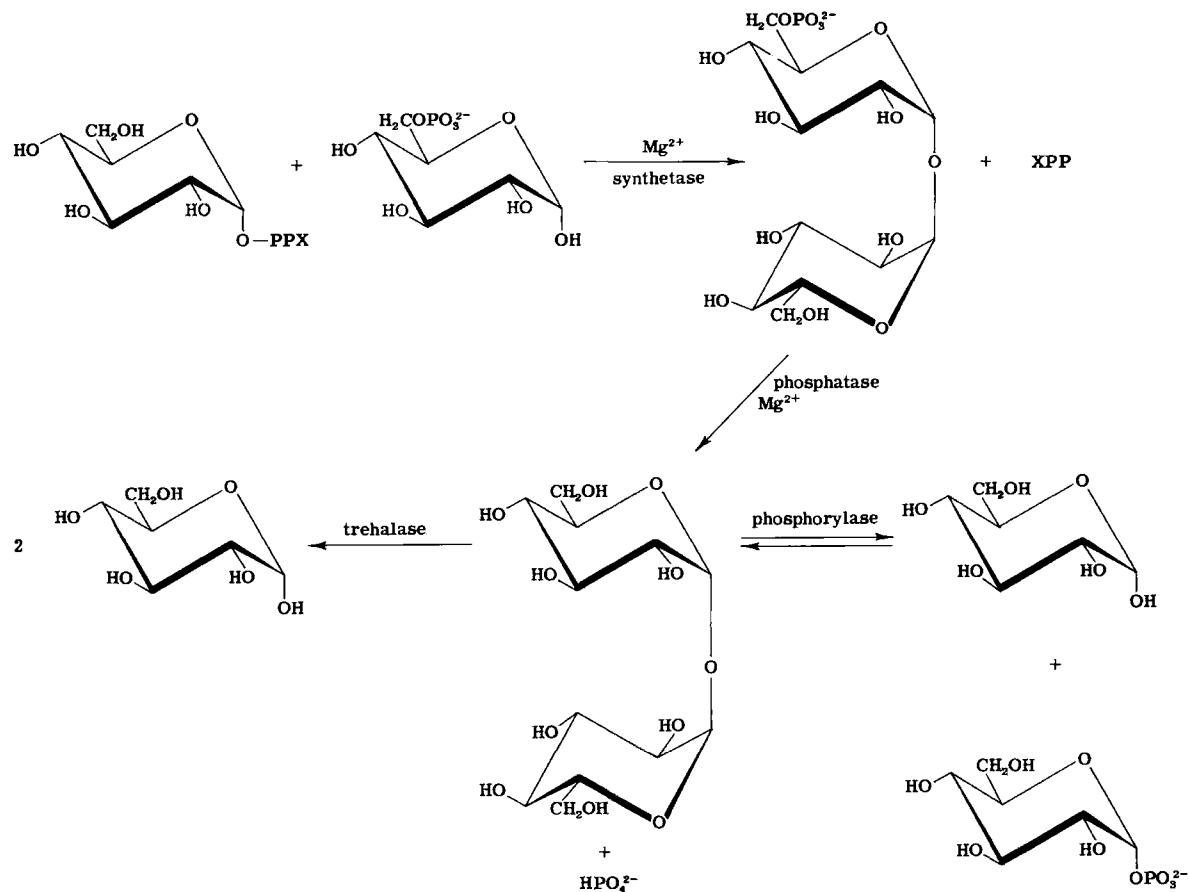


FIG. 1.—Reactions Involved in the Metabolism of α,α -Trehalose. [Synthetase refers to the trehalose 6-phosphate synthetase (XDP-D-glucose:D-glucose 6-phosphate 1-D-glucosyl transferase), phosphatase to the trehalose 6-phosphate phosphatase, and phosphorylase to the α,α -trehalose phosphorylase.]

α,α -trehalose, as a considerable amount of information is available on this subject.

Figure 1 outlines the various reactions that have been shown to be involved in the metabolism of trehalose. Each of the enzymes involved will be discussed in terms of its distribution, localization, properties, and possible regulation.

The other isomers of trehalose containing D-glucopyranose, that is, α,β -trehalose¹ and β,β -trehalose² have been synthesized chemically. However, except for a few rare cases, these isomers of trehalose do not appear to be naturally occurring. Other isomers of trehalose are also structurally possible, such as those containing furanose forms of both sugar moieties, or those containing a pyranose and a furanose, but these have not been found to date. An excellent article on the chemistry of the trehaloses has been written by Birch.³

II. α,α -TREHALOSE

1. Isolation and Identification

α,α -Trehalose is readily extracted from cells and tissues by suspending the material in question in 70% ethanol and bringing the mixture to the boil. After the suspension has been cooled, the precipitate is removed, and the trehalose in the supernatant liquor can be isolated by paper chromatography,⁴ or it can be crystallized directly if the concentration is high enough. α,α -Trehalose is very soluble in water and in aqueous ethanol, but crystallizes readily from solutions containing 80% or more of ethanol. It generally crystallizes as the dihydrate, whose physical properties (m.p. 96–97°, $[\alpha]_D +177$ –182°) are quite distinct from those of the α,β and β,β isomers. A useful derivative of α,α -trehalose is the octaacetate (m.p. 97–98°, $[\alpha]_D +163$ °). Trehalose can also be identified in microamounts by the use of a highly specific trehalase (see p. 246) in combination with D-glucose oxidase. The preparation and partial purification of trehalase from the bacterium *Streptomyces hygroscopicus* is fairly straightforward, and the resulting enzyme-preparation is relatively stable.⁵ Therefore, this enzyme (or a similar preparation from some other source) is useful as an analytical reagent.

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TABLE I
Occurrence of α,α -Trehalose

Group	Specific organism	References
A. Plants		
<i>Pteridophytes</i> (ferns)	<i>Selaginella</i> species	7-9
	<i>Botrychium lunaria</i>	10
<i>Spermatophytes</i> (seed plants)	<i>Echinops persicus</i>	11
	<i>Carex brunescens</i>	12
	<i>Fagus sylvatica</i>	13
B. Algae		
<i>Cyanophyceae</i> (unicellular)	<i>Rivularia bullata</i>	14
<i>Rhodophyceae</i>	<i>Bangia fuscopurpurea</i>	15
	<i>Chondrus crispus</i>	15
	<i>Porphyra laciniata</i>	15
	<i>Cystoclonium purpurescens</i>	15
	<i>Furcellaria fastigiata</i>	15
	<i>Rhodymenia palmata</i>	15
	<i>Lamenea nodosa</i>	16
	<i>Batrachospermum</i>	17
	<i>Serraticardia marina</i>	18
C. Eumycetes (Fungi and Yeasts)		
<i>Myxomycetes</i>	<i>Dictyostelium discoideum</i>	19
	<i>Dictyostelium mucoroides</i>	20
<i>Ascomycetes</i>	<i>Neurospora tetrasperma</i>	21
	<i>Claviceps purpurea</i> (ergot of rye)	6,22
	<i>Saccharomyces cerevisiae</i>	23
<i>Basidiomycetes</i>	<i>Puccinia graminis</i>	24
	<i>Fomes fomentarius</i>	25
	<i>Fomes annosus</i>	25
<i>Fungi imperfecti</i>	<i>Pullularia pullulans</i>	26
	<i>Penicillium chrysogenum</i>	27
	<i>Aspergillus luchuensis</i>	28
	<i>Myrothecium verrucaria</i>	28
	<i>Pithomyces chartarum</i> ^a	29
	<i>Sclerotium cepivorum</i>	30
	<i>Helminthosporium sativum</i>	31
<i>Mycorrhiza</i>	mycorrhizal roots of beech	32
D. Schizomycetes (Bacteria)		
<i>Actinomycetes</i>	<i>Mycobacterium tuberculosis</i>	33-35
	Other mycobacteria	36,37
	<i>Nocardia</i> species	38
	<i>Streptomyces</i> species	4
Other bacteria	<i>Corynebacteria</i> species	39,40

TABLE I (Continued)

Group	Specific organism	References
<i>E. Arthropods (Insects)</i>		
<i>Orthoptera</i>		
House cricket	<i>Gryllus domesticus</i>	41
Cockroach	<i>Leucophaea maderae</i>	42
Grasshopper	<i>Melanophus differentialis</i>	43
Cockroach	<i>Periplaneta americana</i>	44,45
Locust	<i>Schistocerca gregaria</i>	46,47
<i>Homoptera</i>		
Aphid	<i>Megoura viciae</i>	48
<i>Lepidoptera</i>		
Silkworm	<i>Antheraea pernyi</i>	49
	<i>Antheraea polyphemus</i>	50
	<i>Bombyx mori</i>	50,51
Hawk moth	<i>Celerio euphorbiae</i>	52,53
	<i>Deilephila elpenor</i>	54
Wax moth	<i>Galleria mellonella</i>	50
	<i>Hyalophora cecropia</i>	50
Armyworm	<i>Leucania separata</i>	55
Eri (silkworm)	<i>Samia cynthia</i>	55,56
	<i>Sphinx ligustri</i>	54
Tobaccoworm	<i>Protoperce sexta</i>	50
Silk moth	<i>Telea polyphemus</i>	50
	<i>Platysamia cecropia</i>	50
<i>Coleoptera</i>		
Water beetle	<i>Chalcophora mariana</i>	54
	<i>Dytiscus marginalis</i>	54
	<i>Ergates faber</i>	54
	<i>Hydrous piceus</i>	54,57
Mealworm	<i>Tenebrio molitor</i>	54,58
Flour beetle	<i>Tribolium confusum</i>	50
<i>Hymenoptera</i>		
Honeybee	<i>Anthophora</i> sp.	54
	<i>Apis mellifera</i>	54,59
Sawfly	<i>Diprion hercyniae</i>	50
	<i>Trichiocampus populi</i>	60
<i>Diptera</i>		
Horse bot-fly	<i>Gastrophilus intestinalis</i>	60
Blowfly	<i>Phormia regina</i>	61-63
Mosquito	<i>Aedes aegypti</i>	50
Fruit fly	<i>Drosophila repleta</i>	50
<i>Hemiptera</i>		
Milkweed bug	<i>Oncopeltus fasciatus</i>	50
<i>F. Other Invertebrates</i>		
Parasitic worm	<i>Ascaris lumbricoides</i>	64,65
Lobster	<i>Homarus americanus</i>	66
	<i>Carcinus maenas</i>	67

(Continued)

TABLE I (Continued)

Group	Specific organism	References
Shrimp	<i>Artemia salina</i>	68
Nematode	<i>Nippostrongylus brasiliensis</i>	69
Helminth	<i>Moniliformis dubius</i>	70
Annelid		
Sandworm	<i>Diopatra cupreae</i>	70
	<i>Glycera dibranchiata</i>	70
	<i>Harmothoe imbricata</i>	70
Earthworm	<i>Lumbricus terrestris</i>	70
Leech	<i>Dina fervida</i>	70
	<i>Glassosiphonia complanata</i>	70
	<i>Hellobdella stagnalis</i>	70
Peanut worm	<i>Golfingia gouldii</i>	70

* Found in small proportions only.

2. General Occurrence of α,α -Trehalose

α,α -Trehalose was probably first isolated from the ergot of rye by Wiggers⁶ in 1832. Since that time, it has been found in a large number of different types of organism, as shown in Table I. For example, in lower

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plants, several pteridophytes (ferns), notably *Selaginella*⁷⁻⁹ and *Botrychium lumaria*,¹⁰ have been shown to contain trehalose. The presence of trehalose in spermatophytes (seed plants) has now been proved unequivocally. In 1858, Berthelot¹¹ isolated trehalose from *Echinops persica*, a species belonging to the composites. However, this sugar apparently came from "Trehalamanna," the cocoons of a beetle (*Larinus*) that is a parasite on this plant. Von Lippmann¹² found trehalose in an exudate of *Carex brunescens* (a member of the sedge family), but was not able to isolate it from the plant itself. These results suggested that trehalose in the exudate might have arisen from microbial activity. However, Oesch and Meier¹³ have isolated trehalose from the cambial sap of the beech tree, *Fagus silvatica*. Their experiments appeared to exclude the possibility that the trehalose arose from microbial contamination.

α,α -Trehalose has also been isolated from several groups of algae, particularly the unicellular cyanophytes,¹⁴ and a number of rhodophytes (red algae).¹⁵⁻¹⁸ This sugar is also very common in fungi and yeasts, for which there is considerable evidence to indicate that it serves as a source of reserve energy (see p. 252). Thus, it has been isolated from myxomycetes,^{19,20} ascomycetes,²¹⁻²³ basidiomycetes,^{24,25} and a number of fungi imperfecti,²⁶⁻³¹ as well as from fungi (mycorrhiza) that infect the roots of beech trees.³² In many fungi, trehalose is found both in the spores and the mycelia, although the levels may vary markedly with the stage of development. Trehalose is also found in some bacteria, but seems to be limited to those so-called "higher bacteria" considered to be closely related to the fungi, that is, the actinomycetes. Thus, free trehalose is found in a number of streptomycetes,⁴ where it occurs not only in the mycelia but also in the spores. It is also found in other actinomycetes, such as mycobacteria³³⁻³⁷ and nocardia,³⁸ and in corynebacteria.³⁹⁻⁴⁰ However, in these organisms, most of the trehalose is esterified with long-chain fatty acids, and only small proportions of free trehalose are found (see p. 235). It seems likely that, in these instances, the free trehalose may be a precursor to the esterified trehalose.

In the animal kingdom, trehalose occurs in a great many arthropods, as shown in Table I. Indeed, this sugar has apparently been found in every species of insect that has been examined; for example, in crickets,⁴¹ cockroaches,^{42,44,45} grasshoppers,⁴³ locusts,^{46,47} aphids,⁴⁸ silkworms,^{49-51,54} moths,⁵²⁻⁵⁴ tobacco worms,⁵⁰ armyworms,⁵⁵ mealworms,^{54,58} beetles,^{54,57} honeybees,^{54,59} sawflies,^{50,60} bot-flies,⁶⁰ blowflies,⁶¹⁻⁶³ fruit flies,⁵⁰ mosquitoes,⁵⁰ and milkweed bugs.⁵⁰ It has also been found in a variety of

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(70) D. Fairbairn, *Can. J. Zool.*, **36**, 787 (1958).

other invertebrates, including nematodes,⁶⁴ parasitic worms,⁶⁵ crustaceans,⁶⁶⁻⁶⁹ and various annelids.⁷⁰ In insects, it is the major sugar found in the blood (hemolymph), and it occurs in both the pupal and the larval stages, as well as in the adult. In adult flies, trehalose functions as the energy source to sustain flight. The levels of trehalose may vary with the developmental stage of the organism.

Asahina and Tanno⁷¹ found that overwintering, pre-pupal larvae of the sawfly *Trichiocampus populi* are able to survive freezing at -40° . These organisms contain a very high concentration of trehalose, ranging from 5-9%, but no glycerol. As frost-resistance in other organisms has been attributed to high concentrations of glycerol or D-glucitol, it seems possible that trehalose may function in an analogous way in this organism.

3. Naturally Occurring Derivatives of α,α -Trehalose

Much of the trehalose found in mycobacteria (and probably in nocardia and corynebacteria) does not occur as free trehalose, but as a derivative. Thus, the toxic component of the cell walls of *Mycobacterium tuberculosis*, called "cord factor," is a glycolipid, namely, the α,α -trehalose 6,6'-diester of mycolic acid [$C_{60}H_{120}(HO)-CHOH-CH-(C_{24}H_{49})CO_2H$].^{33,34} Trehalose dimycolates have also been isolated from a number of other mycobacteria.^{36,37} Although similar types of glycolipids are found in other closely related organisms, such as nocardia³⁸ and corynebacteria,^{39,40} the trehalose therein is esterified with other fatty acids (nocardic and corynomycolic acids) instead of mycolic acid.⁷² In reference to the biological activity of these materials, aqueous emulsions of cord factor in oil produce, upon intravenous injection, a granulomatous response in the lungs of mice. The granules have the appearance of tubercles, and are indistinguishable from those formed after an infection^{73,74} with living *Bacillus Calmette-Guerin*. Cord factor alone is not antigenic, but Kato⁷⁵ prepared immunogenic complexes of cord factor with methylated bovine serum-albumin. Mice vaccinated subcutaneously with the complex in incomplete Freund's adjuvant exhibited high resistance against both small multiple and large single injections of the toxic glycolipid.

Mycobacterium tuberculosis also produces several other trehalose-

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(74) A. Bekierhunst, E. Yarkoni, I. Flechner, S. Morecki, E. Vilkas, and E. Lederer, *Infect. Immun.*, **4**, 256 (1971).

(75) M. Kato, *Amer. Rev. Resp. Dis.*, **98**, 668 (1969).

containing compounds. Middlebrook and coworkers⁷⁶ isolated a sulfated glycolipid from strain H37Rn. Goren^{77,78} found that several families of related sulfolipids are produced by *Mycobacterium tuberculosis*, the principal sulfatide of which is a 2,3,6,6'-tetra-*O*-acyltrehalose 2-sulfate. Three different acyl substituents were found in these compounds, namely, those of hexadecanoic (palmitic) acid, 17-hydroxy-2,4,6,8,10,12,14,16-octamethyldotriacontanoic (hydroxyphthioceranic) acid, and 2,4,6,8,10,12,14-heptamethyltriacontanoic (phthioceranic) acid. Narumi and Tsumita³⁵ isolated from *M. tuberculosis* a phosphorylated polysaccharide containing trehalose. The main component was a polymer of α,α -trehalose 6,6'-diphosphate having side chains of D-mannose linked through the phosphate group.

A *Streptomyces* species has been shown by Arcamone and Bizioli⁷⁹ to produce 2-amino-2-deoxy- α -D-glucopyranosyl α -D-glucopyranoside (α,α -trehalosamine), and to excrete this amino disaccharide into the culture medium. Interestingly, this compound exhibits antibiotic activity against mycobacteria, probably because it is a competitive inhibitor of the trehalase.⁸⁰

The distribution of α,α -trehalose 6-phosphate is not really known, as its isolation has only been reported for a few organisms. However, it seems almost certain that this compound occurs, albeit in small proportion, in all organisms that synthesize trehalose, because the 6-phosphate is an intermediate in the biosynthesis of trehalose (see p. 237). However, α,α -trehalose 6-phosphate was first isolated from yeast by Robison and Morgan⁸¹ in 1928. It has also been found in insects,⁸² and in spores of streptomycetes.⁸³ α,α -Trehalose 6-phosphate was chemically synthesized by MacDonald and Wong,⁸⁴ by phosphorylation of α,α -trehalose with a stoichiometric proportion of diphenylphosphorochloridate, followed by acetylation, hydrogenation in the presence of Adams catalyst, and deacetylation. The resulting mixture of α,α -trehalose monophosphate and α,α -trehalose diphosphate was separated on a column of Dowex-1 (formate) ion-exchange resin.

(76) G. Middlebrook, C. Coleman, and W. B. Schaefer, *Proc. Nat. Acad. Sci. U. S.*, **45**, 1801 (1959).

(77) M. B. Goren, *Biochim. Biophys. Acta*, **210**, 116 (1970).

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(79) F. Arcamone and F. Bizioli, *Gazz. Chim. Ital.*, **87**, 896 (1957).

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(81) R. Robison and W. T. J. Morgan, *Biochem. J.*, **22**, 1277 (1928).

(82) S. Friedman, *Arch. Biochem. Biophys.*, **88**, 339 (1960).

(83) A. H. Ferguson, M. Mitchell, and A. D. Elbein, *J. Bacteriol.*, **116**, 1084 (1973).

(84) D. L. MacDonald and R. Y. K. Wong, *Biochim. Biophys. Acta*, **86**, 380 (1964).

M. Fischer and Kandler⁸⁵ isolated from *Selaginella kraussiana* a non-reducing trisaccharide which they named selaginose; it was characterized as 2-O- α -D-glucopyranosyl- α,α -trehalose. Farkaš and coworkers⁸⁶ isolated a nonreducing disaccharide from yeasts grown on 2-deoxy-D-arabino-hexose. The disaccharide was alkali-stable, but very acid-labile, and was tentatively identified as 2,2'-dideoxy- α,α -trehalose (2-deoxy- α -D-arabino-hexopyranosyl 2-deoxy- α -D-arabino-hexopyranoside).

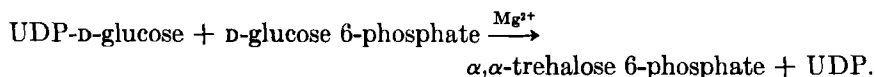
III. NATURAL OCCURRENCE OF OTHER ISOMERS OF TREHALOSE

As already indicated, α,α -trehalose is widespread, whereas the other isomers of this disaccharide are virtually unknown in Nature. However, W. Fischer and Krieglstein⁸⁷ isolated 3-O- α -D-glucopyranosyl- α,β -trehalose from *Streptococcus faecalis*. This trisaccharide is present in substantial proportion in these cells, because 5 g of it could be isolated from 200 g (wet weight) of bacteria, but its function is not yet known.

IV. MECHANISMS OF BIOSYNTHESIS OF α,α -TREHALOSE

1. From Uridine 5'-(α -D-Glucopyranosyl Pyrophosphate)

The mechanism of biosynthesis of α,α -trehalose was first demonstrated by Cabib and Leloir⁸⁸ in brewers' yeast as shown by the following reaction:



By use of a cell-free extract from the yeast, these workers were able to show the transfer of D-glucose from the "sugar nucleotide" UDP-D-glucose, to D-glucose 6-phosphate to form the 6-phosphate of α,α -trehalose and uridine 5'-pyrophosphate (UDP). The enzyme showed maximal activity at pH 6.6 in the presence of 25 mM Mg^{2+} . The equilibrium of the reaction was strongly in favor of synthesis of trehalose phosphate, and reversibility could not be demonstrated. In a subsequent step, the phosphate group was removed by a phosphatase (see p. 245). to give free trehalose, which is apparently then stored in large amounts.

(85) M. Fischer and O. Kandler, Abstract; Trehalose Day Symposium, Paris, France (1970).

(86) V. Farkaš, Š. Bauer, and J. Zemek, *Biochim. Biophys. Acta*, **184**, 77 (1969).

(87) W. Fischer and J. Krieglstein, *Z. Physiol. Chem.*, **348**, 1252 (1967).

(88) E. Cabib and L. F. Leloir, *J. Biol. Chem.*, **231**, 259 (1968).

Panek⁸⁹ reported similar findings with extracts of bakers' yeast, but detected trehalose, not trehalose 6-phosphate; however, this may have been due to the presence of a highly active phosphatase. Elander⁹⁰ studied the trehalose 6-phosphate synthetase of yeast after purifying the enzyme about 27-fold. The synthesis of trehalose 6-phosphate was inhibited by uridine 5'-pyrophosphate, which was competitive with UDP-D-glucose, and also by uridine 5'-triphosphate (UTP). It was also inhibited by 20 mM concentrations of inorganic phosphate, D-glucosyl phosphate, D-fructose 6-phosphate, D-mannose 6-phosphate, and trehalose 6-phosphate. D-Mannose 6-phosphate and trehalose 6-phosphate were competitive with D-glucose 6-phosphate. Free trehalose had no effect on the reaction.

Treherne⁹¹ performed some *in vivo* experiments with the locust, *Schistocerca gregoria*, and found that introduction of D-[¹⁴C]glucose either into the alimentary canal, or directly into the hemolymph, resulted in the rapid appearance of radioactive trehalose. Several years later, Candy and Kilby⁹² found that cell-free extracts prepared from the fat body (a tissue somewhat similar to mammalian liver) of locusts catalyzed the synthesis of radioactive trehalose from D-[¹⁴C]glucose in the presence of adenosine 5'-triphosphate (ATP) and UDP-D-glucose. Presumably, the extract contained hexokinase and phosphoglucomutase, as well as the trehalose phosphate synthetase (UDP-D-glucose:D-glucose 6-phosphate 1-D-glucosyl transferase), so that the synthesis of trehalose 6-phosphate appeared to be analogous to that described for yeast. However, in this work, the trehalose 6-phosphate synthetase was not further purified, so that its absolute requirements are not known.

The UDP-D-glucose:D-glucose 6-phosphate 1-D-glucosyl transferase was also demonstrated in the fat bodies of silkmooths by Murphy and Wyatt.⁹³ These authors found that the enzyme is located in the soluble portion of the cytoplasm, and that it has two unusual properties. Firstly, the synthesis of trehalose phosphate from UDP-D-glucose and D-glucose 6-phosphate was inhibited by the addition of free trehalose; and secondly, the kinetics of formation of trehalose phosphate with respect to D-glucose 6-phosphate are complex and sigmoidal, indicative of an allosteric effect. The sigmoidal nature of the kinetics with D-glucose 6-phosphate was abolished when the enzyme was precipitated with ammonium sulfate, or when mercuric acetate was added to the incubation mixtures.

(89) A. Panek, *Arch. Biochem. Biophys.*, **98**, 349 (1962).

(90) M. Elander, *Ark. Kemi*, **31**, 17 (1968).

(91) J. E. Treherne, *J. Exp. Biol.*, **35**, 611 (1958).

(92) J. Candy and B. A. Kilby, *Biochem. J.*, **78**, 531 (1961).

(93) T. A. Murphy and G. R. Wyatt, *J. Biol. Chem.*, **240**, 1500 (1965).

Their results⁹³ suggested that trehalose may bind to the enzyme at a site other than the active site, and thereby inhibit enzymic activity. This type of feedback inhibition could be important in regulating the levels of trehalose in the blood of these insects. Friedman⁹⁴ found that trehalose also inhibits the enzyme in the blowfly *Phormia regina*.

Fisher⁹⁵ incubated extracts of the parasitic worms *Moniliformis dubius* and *Macracanthorhynchus* with D-[¹⁴C]glucose in the presence of ATP and UDP-D-glucose, and was able to show the formation of radioactive trehalose. Much less radioactivity was found in the trehalose when either UDP-D-glucose or ATP was omitted from the reaction mixtures. McAlister and Fisher⁹⁶ further examined the synthesis of trehalose in extracts of *Moniliformis dubius*. They found that the synthetase activity was located in the body wall of adult female worms, but could not detect activity in body fluid or acanthors (a stage in the life cycle of *Acanthocephala*). Like the yeast and insect enzymes, the synthetase was specific for UDP-D-glucose as the D-glucosyl donor. The addition of trehalose at 30 mM concentration caused a 50% inhibition in activity, suggesting that trehalose may control its own synthesis. Presumably, the product of the synthetase reaction is trehalose 6-phosphate, although this compound was not identified. Feist and coworkers⁹⁷ found that reproductive tissue from adult worms of *Ascaris suum* incorporated D-[¹⁴C]glucose into trehalose. Limited synthesis of trehalose occurred with muscle tissue. Presumably, the synthesis of trehalose in these worms occurs by the same mechanism as in other parasites.

Schwoch⁹⁸ studied the synthesis of trehalose in the crayfish, *Orconectes limosus*. Biosynthesis of the disaccharide could be detected in the abdominal muscle, antennal gland, hind-gut, integumentary tissue, and, to a lesser extent, in the stomach tissue. The trehalose 6-phosphate synthetase was soluble and was inhibited 50% by 80 mM trehalose. The rate of synthesis of trehalose was dependent on the season, and optimal activity in abdominal muscle occurred in February and March. The label from injected [¹⁴C]trehalose or D-[¹⁴C]glucose was found to be distributed from the hemolymph into organs much faster than the label from [¹⁴C]maltose.

The biosynthesis of trehalose has also been studied during various stages of development, especially in the cellular slime-mold *Dictyostelium*

(94) S. Friedman, *J. Insect Physiol.*, **13**, 397 (1967).

(95) F. M. Fisher, Jr., *J. Parasitol.*, **50**, 803 (1964).

(96) R. O. McAlister and F. M. Fisher, Jr., *J. Parasitol.*, **58**, 51 (1972).

(97) C. F. Feist, C. P. Read, and F. M. Fisher, Jr., *J. Parasitol.*, **51**, 76 (1965).

(98) G. Schwoch, *Comp. Biochem. Physiol.*, **43**, 905 (1972).

discoideum. The synthesis of trehalose 6-phosphate from UDP-D-glucose in cell-free extracts of this organism was first demonstrated by Roth and Sussman.⁹⁹ These workers also examined the activity of the trehalose 6-phosphate synthetase at various stages of development.¹⁰⁰ The enzyme could not be detected in vegetative cells (solitary amebas) of *Dictyostelium discoideum*, but it began to accumulate about 5 hours after culmination had begun (that is, 5 hours after the start of morphogenesis). The enzymic activity reached a peak at 16 hours, and then began to decline. Inhibitors of protein synthesis, such as 3-[2-(dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide (cycloheximide), prevented increases in enzymic activity, indicating that the increase in activity was due to new protein synthesis. Various mutant strains blocked at various stages of development were also examined with regard to the trehalose 6-phosphate synthetase. Those mutants blocked at early stages of development accumulated no enzyme, whereas those blocked at later stages accumulated some synthetase, but the enzyme activity did not decline as it did in the wild types.

Newell and coworkers¹⁰¹ found that, during particular stages of fruit construction in *Dictyostelium discoideum*, four functionally related enzymes accumulated, reached characteristic levels of specific activity, and then disappeared partly or completely. These enzymes were UDP-D-glucose pyrophosphorylase, trehalose 6-phosphate synthetase, UDP-D-galactose 4-epimerase, and UDP-D-galactose:polysaccharide D-galactosyl transferase.

Based on known changes in the concentration of trehalose, the K_m values of UDP-D-glucose pyrophosphorylase for UTP and D-glucosyl phosphate, and the rate of synthesis of UDP-D-glucose *in vivo*, Wright and R. Marshall¹⁰² constructed a kinetic model to simulate the accumulation of trehalose during the differentiation of *Dictyostelium discoideum*. Their model predicted that trehalose synthetase activity *in vivo* does not increase during the same period in which *in vitro* analyses indicate an accumulation of this enzyme. The models also predicted that the rate of synthesis of trehalose *in vivo* should increase very rapidly over a brief period of time, late in culmination, and be of the order of 0.03 μ mole per minute per ml of packed cells. Sargent and Wright¹⁰³ studied the synthesis of trehalose *in vivo* during differentiation of the slime mold, to

(99) R. Roth and M. Sussman, *J. Biol. Chem.*, **243**, 5081 (1968).

(100) R. Roth and M. Sussman, *Biochim. Biophys. Acta*, **122**, 225 (1966).

(101) P. C. Newell, J. Franke, and M. Sussman, *J. Mol. Biol.*, **63**, 373 (1972).

(102) B. E. Wright and R. Marshall, *J. Biol. Chem.*, **246**, 5335 (1971).

(103) D. Sargent and B. E. Wright, *J. Biol. Chem.*, **246**, 5340 (1971).

determine the accuracy of the aforementioned model. Cells at various stages of development were exposed to D-[^{14}C]glucose for short times, and the radioactivity in the trehalose, the UDP-D-glucose, and the D-glucose 6-phosphate was determined. From aggregation until late in the culmination process, the rate of synthesis of trehalose was found to be negligible. At that time, a 100-fold burst of synthetic activity was observed which amounted to 0.04 μmole of trehalose per minute per ml of packed cells. Thus, the experimental data fit the predictions of kinetic models well.

Killick and Wright¹⁰⁴ examined the levels of the enzyme trehalose 6-phosphate synthetase at various stages of development of the slime mold, and obtained results that contradicted those of Roth and Sussman.^{99,100} They found¹⁰⁴ that the enzyme was not only present at pre-culmination, but was also present in a masked form at the beginning of differentiation. By selective fractionation with ammonium sulfate and pre-incubation at 35°, they were able to unmask the enzyme and show its activity. Some evidence was obtained for the presence of an extra-cellular inhibitor of the enzyme.

Gussin and McCormack¹⁰⁵ presented evidence for the presence of trehalose 6-phosphate synthetase in the pollen of *Lilium longiflorum*.

2. From Other D-Glucosyl Esters of Nucleoside Pyrophosphates

Although the most widespread D-glucosyl donor for the synthesis of trehalose 6-phosphate is UDP-D-glucose, other D-glucose "sugar nucleotides" can also serve as D-glucosyl donors in certain organisms. Elbein¹⁰⁶ tested various radioactive D-glucose nucleotides (ADP-D-[^{14}C]glucose, CDP-D-[^{14}C]glucose, GDP-D-[^{14}C]glucose, TDP-D-[^{14}C]glucose, and UDP-D-[^{14}C]glucose) as substrates for synthesis of trehalose 6-phosphate in cell-free extracts of *Streptomyces hygroscopicus*; only GDP-D-[^{14}C]glucose was able to give rise to radioactive trehalose in this system. By a variety of chemical, enzymic, and chromatographic methods, the product formed by the enzyme extract was identified as α,α -trehalose 6-phosphate. The trehalose 6-phosphate synthetase (GDP-D-glucose:D-glucose 6-phosphate 1-D-glucosyl transferase) was purified 100-fold from extracts of *S. hygroscopicus*, and shown to be specific for GDP-D-glucose and D-glucose 6-phosphate as substrates. Trehalose 6-phosphate was not formed by this enzyme when UDP-D-glucose was used as the substrate. Although the enzyme did not exhibit an absolute requirement for Mg^{2+} , this cation

(104) K. A. Killick and B. E. Wright, *J. Biol. Chem.*, **247**, 2967 (1972).

(105) A. E. S. Gussin and J. H. McCormack, *Phytochemistry*, **9**, 1915 (1970).

(106) A. D. Elbein, *J. Biol. Chem.*, **242**, 403 (1967).

did stimulate enzymic activity.¹⁰⁷ In addition to *S. hygroscopicus*, the GDP-D-glucose-dependent trehalose 6-phosphate synthetase has been found in a number of other streptomycetes, indicating that this group of organisms synthesizes trehalose 6-phosphate by a mechanism different from that previously described.⁴

Goldman and Lornitzo¹⁰⁸ studied the biosynthesis of trehalose 6-phosphate in *Mycobacterium tuberculosis*, an organism closely related to the streptomycetes. Cell-free extracts of this organism catalyzed the synthesis of trehalose (trehalose 6-phosphate) from UDP-D-glucose and D-glucose 6-phosphate. These workers¹⁰⁸ found that the specific activity of the trehalose 6-phosphate synthetase was much higher in a virulent strain of the organism than in an avirulent mutant (H37Ra). The low activity in the avirulent mutant was due to the presence, in these crude extracts, of a compound that inhibited the enzyme in a noncompetitive manner. The inhibitor, called mycoribnin, could be separated from the enzyme by isoelectric precipitation, and was found to be an oligoribonucleotide, having a molecular weight of about 4,000, containing adenine and guanine.¹⁰⁹ Lornitzo and Goldman¹¹⁰ found that trehalose 6-phosphate synthetase prepared from young, or old, cultures of H37Ra, as well as the enzyme from brewers' yeast, was sensitive to mycoribnin. However, the sensitivity was lost when these enzymes were treated with millimolar concentrations of bicarbonate. The bicarbonate-induced changes in the enzyme could not be reversed by dilution, but mycoribnin-insensitive enzyme-preparations could be made sensitive by certain enzyme-fractionation procedures. The data are consistent with the notions that bicarbonate and mycoribnin compete for a site on the protein (probably different from the substrate site), and that binding of these molecules alters the conformation of the protein. Presumably, bicarbonate binds the more tightly to the protein, as it cannot be displaced by mycoribnin, whereas bicarbonate can displace mycoribnin.

Although Goldman and Lornitzo¹⁰⁸ showed that the mycobacterial trehalose 6-phosphate synthetase utilized UDP-D-glucose as the D-glucosyl donor, they did not test other D-glucosyl donors, such as GDP-D-glucose, because such "sugar nucleotides" were unavailable at that time. However, somewhat later, Liu and coworkers¹¹¹ found that crude extracts of *Mycobacterium smegmatis* (and of several other mycobacteria) catalyzed

(107) A. D. Elbein, *J. Bacteriol.*, **96**, 1623 (1968).

(108) D. S. Goldman and F. A. Lornitzo, *J. Biol. Chem.*, **237**, 3332 (1962).

(109) F. A. Lornitzo and D. S. Goldman, *J. Biol. Chem.*, **239**, 2730 (1964).

(110) F. A. Lornitzo and D. S. Goldman, *J. Bacteriol.*, **89**, 1086 (1965).

(111) C. Liu, B. W. Patterson, D. Lapp, and A. D. Elbein, *J. Biol. Chem.*, **244**, 3728 (1969).

the synthesis of trehalose from both UDP-D-glucose and GDP-D-glucose. In order to determine whether these extracts contained one, rather non-specific, synthetase, or whether two different enzymes were present, the crude, *M. smegmatis* enzyme-preparation was placed on a column of DEAE-cellulose, in order to separate and purify the various enzymes. From the DEAE-cellulose column was eluted a protein fraction that had activity with GDP-D-glucose, but no UDP-D-glucose activity was detected. However, when later fractions from the column were added to this protein peak, activity with UDP-D-glucose was restored. The active component in these later column-fractions was found to be ribonucleic acid (RNA). Trehalose phosphate synthetase activity with both UDP-D-glucose and GDP-D-glucose appeared to reside in the same protein, as both activities were lost at the same rate when the enzyme fraction was heated at various temperatures.

Lapp and coworkers¹¹² found that a number of polyanions of high molecular weight, especially the sulfated glycosaminoglycans heparin, chondroitin sulfate, heparitin sulfate, and dermatan sulfate, are potent activators of the enzyme. In addition, a number of polynucleotides of high molecular weight were also good effectors. However, such polymers as hyaluronic acid, D-galacturonan, and poly(glutamic acid), whose polyanionic nature is due to the presence of carboxyl groups, are not active in stimulating the enzyme to utilize UDP-D-glucose. The polyanion did not appear to cause activation either by protecting the enzyme or the substrates from degradation, or by binding or removal of products or inhibitors. Since the [³H]guanine-polynucleotide activator isolated from *M. smegmatis* (grown on [³H]guanine) bound to the enzyme, it appears likely that it causes a conformational change in the protein to give a more active form of the enzyme. In addition to utilizing UDP-D-glucose and GDP-D-glucose as substrate, the trehalose phosphate synthetase is also able to use ADP-D-glucose, CDP-D-glucose, and TDP-D-glucose, although there are differences in their relative effectiveness as D-glucosyl donors for trehalose phosphate. However, with CDP-D-glucose and TDP-D-glucose, the enzyme behaves in the same way as with UDP-D-glucose; that is, it requires a polyanion of high molecular weight for activity. On the other hand, the enzyme responds to ADP-D-glucose in the same way as to GDP-D-glucose; formation of trehalose phosphate is stimulated by polyanion, but there is no absolute requirement for this effector. Thus, the enzyme appears to distinguish between "sugar nucleotides" containing a pyrimidine and those containing a purine.

(112) D. Lapp, B. W. Patterson, and A. D. Elbein, *J. Biol. Chem.*, **246**, 4567 (1971).

3. Other Enzymic Mechanisms

It is possible that the trehalose phosphorylase described in Section V,3 (see p. 254) could be used to synthesize trehalose from D-glucosyl phosphate plus D-glucose, because the reaction appears to be freely reversible *in vitro*. However, as most phosphorylases appear to be degradative enzymes, this enzyme is discussed under the catabolism of trehalose (see p. 245).

4. Possible Control Mechanisms

The results of various studies indicate that the synthesis of trehalose is probably under some sort of regulation. Elander⁹⁰ found that, in yeast, high concentrations (20 mM) of trehalose 6-phosphate, as well as other sugar phosphates, inhibit the trehalose 6-phosphate synthetase. Trehalose phosphate was a competitive inhibitor with respect to D-glucose 6-phosphate, and appeared to compete for the substrate binding-site. On the other hand, in higher organisms, such as silkworms,⁹³ blowflies,⁹⁴ and parasitic worms,⁹⁶ high concentrations (30–50 mM) of trehalose were found to inhibit the synthesis of trehalose 6-phosphate. In at least one of these examples,⁹³ the kinetics were sigmoidal, suggesting an allosteric effect and indicating that trehalose might inhibit its own synthesis by feedback. The fact that fairly high concentrations of trehalose (or trehalose 6-phosphate) are required for inhibition is probably not surprising when it is recalled that trehalose serves as a reserve-energy material in these organisms and is, therefore, stored in fairly large amounts. Despite the high concentrations of trehalose stored in these organisms, it still seems logical to assume that there is a control to prevent continued synthesis of trehalose under some conditions.

In mycobacteria, the trehalose phosphate synthetase appears to be insensitive to trehalose or trehalose 6-phosphate. Lapp and Elbein¹¹³ examined the activities of the various enzymes (such as ADP-D-glucose pyrophosphorylase) involved in the synthesis of D-glucose "sugar nucleotides" in *Mycobacterium smegmatis*. High concentrations (10–20 mM) of trehalose 6-phosphate caused a 50% inhibition in the synthesis of GDP-D-glucose (GDP-D-glucose pyrophosphorylase) and ADP-D-glucose (ADP-D-glucose pyrophosphorylase), but had no effect on the synthesis of UDP-D-glucose. The effect was specific for trehalose 6-phosphate, and was not shown by trehalose, trehalose 6,6'-diphosphate, or other sugar phosphates. The partially purified ADP-D-glucose pyrophosphorylase was also sensitive to trehalose 6-phosphate, whereas the UDP-D-glucose pyrophosphorylase was not. Thus, in this instance, syn-

(113) D. Lapp and A. D. Elbein, *J. Bacteriol.*, **112**, 327 (1972).

thesis of trehalose 6-phosphate may to some extent be controlled by the availability of the D-glucosyl donors (ADP-D-glucose and GDP-D-glucose) whose synthesis is inhibited by high concentrations of end product. In this system, synthesis of trehalose 6-phosphate can also occur from UDP-D-glucose and other D-glucosyl pyrimidine nucleotides, but here, the enzyme requires the presence of a polyanion of high molecular weight for activity. Thus, a control mechanism involving polyanion activation of the synthetase and feedback inhibition of the pyrophosphorylases could be postulated.

V. CATABOLISM OF α,α -TREHALOSE

1. α,α -Trehalose 6-Phosphate Phosphatase

a. Distribution, Specificity, and Location.—As already discussed, trehalose is biosynthesized as the 6-phosphate, although it is generally stored as the free sugar. It therefore appears most probable that organisms that store trehalose contain a phosphatase capable of cleaving trehalose 6-phosphate. Indeed, the conversion of trehalose phosphate into trehalose could be a mechanism for ensuring the continued synthesis of trehalose by removal of one of the products. However, the presence of a specific trehalose 6-phosphate phosphatase has only been demonstrated in a few organisms.

Cabib and Leloir⁸⁸ first detected the presence of a specific trehalose 6-phosphate phosphatase during their studies on the biosynthesis of trehalose 6-phosphate in yeast. The partially purified, trehalose 6-phosphate synthetase preparation also contained a specific phosphatase that cleaved trehalose 6-phosphate but did not hydrolyze UDP-D-glucose, UDP, D-glucose 6-phosphate, or D-glucosyl phosphate. Friedman¹¹⁴ purified this enzyme 56-fold from crude extracts of adult blowflies (*Phormia regina*), and tested it against a wide variety of sugar phosphates and other phosphorylated compounds. The enzyme was highly specific for trehalose 6-phosphate, but did hydrolyze D-glucose 6-phosphate at a rate $\sim 8\%$ that of its normal substrate. The enzyme required a divalent cation for activity, being most active with Mg^{2+} , and had a pH optimum of ~ 7.0 .

b. Possible Control Mechanisms.—A highly specific trehalose 6-phosphate phosphatase was also purified about 50-fold from extracts of *Mycobacterium smegmatis* by Matula and coworkers.¹¹⁵ This enzyme also had a pH optimum of 7.0, and was stimulated by Mg^{2+} . The enzyme

(114) S. Friedman, *Methods Enzymol.*, **8**, 372 (1966).

(115) M. Matula, M. Mitchell, and A. D. Elbein, *J. Bacteriol.*, **107**, 217 (1971).

had slight activity with D-mannose 6-phosphate and D-fructose 6-phosphate, but was inactive on a large number of other phosphorylated compounds, including trehalose 6,6'-diphosphate. Citrate was a competitive inhibitor of the enzyme, both with respect to the concentration of trehalose 6-phosphate and the Mg^{2+} concentration. Although this inhibition appeared to be due to the chelation of Mg^{2+} , it could have a physiological significance as a control of phosphatase activity. (Ethylene-dinitrilo)tetraacetic acid (EDTA) and sodium fluoride were also inhibitors, but these inhibitions were noncompetitive.

Other workers studying the biosynthesis of trehalose 6-phosphate in various organisms have suggested the presence of a trehalose 6-phosphate phosphatase, because, in these studies, the product of the biosynthetic reaction was trehalose, not the expected trehalose 6-phosphate.^{89,96,105} However, in these studies, the phosphatase was not actually identified, and, therefore, cleavage of trehalose 6-phosphate to trehalose could be due to a nonspecific phosphatase.

An enzyme that hydrolyzes D-glucose 6-phosphate to D-glucose and inorganic phosphate was purified 86-fold from extracts of *Phormia regina*.¹¹⁶ The hydrolysis of D-glucose 6-phosphate by this enzyme was markedly stimulated by the addition of trehalose to the incubation mixture, and a number of other sugars could not replace trehalose in this capacity. The same enzyme is also capable of hydrolyzing trehalose 6-phosphate, but, here, trehalose has no effect on the reaction. Friedman¹¹⁶ suggested that the hydrolytic sites are overlapping, but not identical, as inorganic phosphate competitively inhibits both activities, whereas D-glucose competitively inhibits hydrolysis of D-glucose 6-phosphate, but has no effect on the hydrolysis of trehalose 6-phosphate; thus, the overlap appears to occur at the phosphate-binding site, whereas the sugar-binding sites are independent. This enzyme also requires Mg^{2+} , and has a pH optimum for D-glucose 6-phosphate of 7.0, and for trehalose 6-phosphate, of 6.0–6.5. Interestingly, in the presence of trehalose, the pH optimum for hydrolysis of D-glucose 6-phosphate is shifted to 6.0.

2. Trehalase

a. Distribution, Specificity, and Location.—The enzyme trehalase (α , α -trehalose 1-D-glucosylhydrolase, EC 3.2.1.28) was first demonstrated in fungi by Bourquelot^{116a} in 1893. Since that time, trehalase has been found in a great variety of different organisms, including some which neither synthesize nor store trehalose. The rationale for the presence of this

(116) S. Friedman, *J. Biol. Chem.*, **246**, 4122 (1971).

(116a) E. Bourquelot, *Bull. Soc. Mycol. Fr.*, **9**, 189, 230 (1893); *Compt. Rend.*, **116**, 826 (1893).

TABLE II
Occurrence and Properties of Trehalase from Various Sources

Source	Location	Substrates attacked (specificity)	pH optimum	K_m^a (trehalose molarity)	References
A. Bacteria					
<i>Pseudomonas fluorescens</i>	intracellular, soluble	trehalose, 6-deoxytrehalose ^b	6.45	20 mM	117
<i>Bacillus cereus</i>	intracellular, soluble, inducible				118
<i>Streptomyces hygroscopicus</i>	intracellular, soluble	trehalose	6.5	20 mM	5
<i>Mycobacterium smegmatis</i>	intracellular, particulate	trehalose, maltose	7.5		119
B. Fungi and Yeasts					
Hybrid yeast	intracellular, soluble	trehalose	6.8-7.0	10 mM	120
<i>Saccharomyces cerevisiae</i>	intracellular, soluble	trehalose, raffinose	5.7	400 μ M	121
<i>Dictyostelium discoideum</i>	intracellular, soluble	trehalose	5.5	1.2 mM	122
<i>Neurospora crassa</i>	ungerminated conidia, intracel- lular, soluble	trehalose	5.5	570 μ M	123,124
<i>Aspergillus oryzae</i>	conidia, intracellular, soluble, "coat- bound"	trehalose	4.0	2.5 mM	125
<i>Myrothecium verrucaria</i>	spores, spore surface		3.7		126
C. Plants					
<i>Lilium longiflorum</i> pollen	soluble		5.4	1 mM	127
<i>Lycopersicon pimpinellifolium</i> (currant tomato)	pollen				128

(Continued)

TABLE II (Continued)

Source	Location	Substrates attacked (specificity)	pH optimum	K_m^a (trehalose molarity)	References
<i>Hemerocallis minor</i> (dwarf, yellow day-lily)	pollen				128
<i>Galtonia candicans</i> (summer hyacinth)	pollen				128
<i>Camellia japonica</i> (camellia)	pollen				128
<i>Lathyrus odoratus</i> (sweet pea)	pollen				128
<i>Saccharum officinarum</i> (sugar cane)	root, leaves, stalks, soluble			100 μM	129
<i>Selaginella martensii</i>	leaf, stem	trehalose	5.7	1.7 mM	130
<i>D. Insects</i>					
<i>Galleria mellonella</i> (wax moth)	larval stage	trehalose	5.5	130 μM	131
<i>Bombyx mori</i> (silkworm)	pupae	trehalose	5.2	440 μM	132
	muscle, midgut tissue	trehalose	6.1	1.7 mM	133
<i>Phormia regina</i> (blowfly)	blood	trehalose	5.6	670 μM	134
<i>Ascaris suum</i>	intestine				135
<i>Hyalophora cecropia</i> (silkworm)	flight muscle, (mem- brane-bound)	trehalose	6.5	3.6 mM	136
<i>Leucophaea maderae</i> (woodroach)	membrane-bound		6.0		137
<i>Blaberus discoidalis</i> (cockroach)	muscle	trehalose	6.0	3 mM	138
<i>Melolontha vulgaris</i> (may-bug)	soluble	trehalose	6.25	600 μM	139
<i>Sarcophaga barbata</i> (fleshfly)	blood-soluble, particulate- mitochondrial		5.3	1.4 mM	140
<i>Formica polyctena</i> (ant)	labial gland, particulate	trehalose	6.1		141
<i>Apis mellifera</i> (honeybee)	particulate		6.5	1.6 mM	142

<i>Manduca sexta</i> (tobacco hornworm)	larvae		6.0	60 mM	143
<i>Melanoplus differentialis</i> (grasshopper)	adult		5.0	5 mM	144
<i>Philosamia ricini</i> (eri; silkworm)	adult				145
<i>Drosophila melanogaster</i> (fruitfly)	adult		5.6	1.8 mM	146
<i>Calliphora erythrocephala</i> (blowfly)	adult	trehalose	5.5-6.3	3.2 mM	147
<i>E. Mammals</i>					
Monotremes					
<i>Tachyglussus aculeatus</i> (echidna)	intestine				148
Marsupials					
<i>Macropus giganteus</i> (grey kangaroo)	intestine				148
<i>Phascogaleos cinereus</i> (koala)	intestine (low levels)				148
<i>Antichinus stuartii</i> (marsupial mouse)	intestine				148
<i>Dasyurus maculatus</i> (tiger cat)	intestine				148
<i>Perameles nasuta</i> (long-nosed bandicoot)	intestine				148
<i>Isodon obesulus</i> (short-nosed bandicoot)	intestine				148
<i>Pseudocheirus perigrinus</i> (ringtail opossum)	intestine				148
<i>Trichosurus vulpecula</i> (brushtail opossum)	intestine				148
Pig	intestine (proximal part)	trehalose	6.0	3 mM	149
Rat	intestine	trehalose			150,151
Rabbit (also found in cat, dog, goat, guinea pig, hamster, man, rat, rhesus monkey)	kidney (renal cortex)	trehalose	6.3	3.5 mM	152
	membrane-bound				
Man	kidney (brush border)		5.7-5.8	3 mM	153
	urine	(presumably similar to kidney trehalase)			154
	serum		5.4		155
					156

^a K_m is the apparent affinity constant of the enzyme for trehalose. K_i is the dissociation constant of the enzyme-inhibitor complex.

^b A blank space indicates that that parameter was not determined.

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- (135) C. F. Feist, C. P. Read, and F. M. Fisher, Jr., *J. Parasitol.*, **51**, 76 (1965).
- (136) A. E. S. Gussin and G. R. Wyatt, *Arch. Biochem. Biophys.*, **112**, 626 (1965).
- (137) E. C. Zebe and W. H. McShan, *J. Cell. Comp. Physiol.*, **53**, 21 (1959).
- (138) A. R. Gilby, S. S. Wyatt, and G. R. Wyatt, *Acta Biochim. Pol.*, **14**, 83 (1967).
- (139) E. Guilloux, J. E. Courtois, and F. Percheron, *Bull. Soc. Chim. Biol.*, **50**, 1915 (1968).
- (140) A. N. Clements, J. Page, K. Borch, and A. J. J. van Ooyen, *J. Insect Physiol.*, **16**, 1389 (1970).
- (141) R. Paulsen, *Arch. Biochem. Biophys.*, **142**, 170 (1971).
- (142) Y. A. Lefebvre and R. E. Huber, *Arch. Biochem. Biophys.*, **140**, 514 (1970).
- (143) D. L. Dahlman, *Insect Biochem.*, **2**, 143 (1972).
- (144) R. F. Derr and D. D. Randall, *J. Insect Physiol.*, **12**, 1105 (1966).
- (145) R. Pant and I. D. Morris, *Experientia*, **36**, 145 (1974).
- (146) G. A. Marzluf, *Arch. Biochem. Biophys.*, **134**, 8 (1969).
- (147) H. Duve, *Insect Biochem.*, **2**, 445 (1972).
- (148) K. R. Kerry, *Comp. Biochem. Physiol.*, **29**, 1015 (1969).
- (149) J. A. Stevens and D. E. Kidder, *Brit. J. Nutrition*, **28**, 129 (1972).
- (150) A. Dahlqvist, *Acta Chem. Scand.*, **14**, 9 (1960).
- (151) A. Dahlqvist and A. Brun, *J. Histochem. Cytochem.*, **10**, 294 (1962).
- (152) B. Sacktor, *Proc. Nat. Acad. Sci. U. S.*, **60**, 1007 (1968).
- (153) J. F. Demelier and J. E. Courtois, *Bull. Soc. Chim. Biol.*, **50**, 2553 (1968).
- (154) J. Labat, C. Bark, J. E. Courtois, and J. E. Demelier, *Ann. Biol. Clin. (Paris)*, **30**, 253 (1972).
- (155) J. E. Courtois, *Biochim. Appl.*, **15**, 171 (1968).
- (156) E. van Handel, *Clin. Chim. Acta*, **29**, 349 (1970).

enzyme in the latter organisms is that they probably ingest trehalose in their diet.

Table II lists some of the sources where trehalase has been found, and some of the properties of these diverse enzyme-preparations. The properties of these different enzymes vary dramatically; some have a pH optimum as low as 3.7, whereas others are most active at pH 7–7.5. In addition, the K_m value for trehalose may be as low as 400 μM or as high as 20 mM. Finally, some of the trehalases appear to be soluble enzymes, whereas others are apparently membrane-bound. Presumably, these properties reflect the role of the particular enzyme in the metabolism of a given organism.

Instead of discussing the properties of each of the enzymes listed in Table II, only certain unusual features or points of particular interest will be mentioned here. The trehalase from *Mycobacterium smegmatis* is bound to the membrane, but can be solubilized by treatment of the particles with 10% aqueous butanol. After solubilization, the enzyme shows a requirement for Mg^{2+} , with the optimal concentration¹¹⁹ at ~ 2.5 mM. The trehalase in the conidia of *Aspergillus oryzae* exists in both a soluble and a “coat-bound” form, and both of these enzymes are competitively inhibited¹²⁵ by D-mannitol. D-Mannitol is also a reserve material in this organism. In *Neurospora crassa*, Chang and Trevithick¹²⁴ found that 25% of the total, cellular invertase and trehalase was located in a purified wall-preparation; this activity was not released by detergents or other chemical reagents, but was released by snail-gut juice or a (1 \rightarrow 3)- β -D-glucanase from *Bacillus circulans*. Trehalase has also been found in the particulate fraction of cell extracts (that is, “membrane-bound”) of various other organisms, including moths,¹³⁶ woodroaches,¹³⁷ blow fly,¹³⁴ honeybee,¹⁴² and fleshfly.¹⁴⁰ In the last organism, the enzyme has been localized in the mitochondrial fraction, firmly bound to the mitochondrial membrane, whereas the other particulate trehalases may be associated with plasma membranes.

Various workers have detected, in addition to soluble and particle-bound trehalases, multiple forms of trehalase in some organisms. Thus, Hill and Sussman¹²³ found several peaks of trehalase activity when extracts of *Neurospora crassa* were purified on columns of DEAE-cellulose. These various peaks were similar with respect to substrate specificity and the effect of inhibitors. Multiple forms of trehalase have also been found in blowflies, by Friedman and Alexander,¹⁵⁷ and in silkworm, by Saito.¹³²

Some trehalases have been found to be inhibited by certain metabo-

(157) S. Friedman and S. Alexander, *Biochem. Biophys. Res. Commun.*, **42**, 818 (1971).

lites; an example is the inhibition of *Aspergillus oryzae* trehalase by D-mannitol.¹²⁵ Yeast trehalase is inhibited by D-glucose and by trehalose 6-phosphate.¹²⁰ The inhibition by trehalose 6-phosphate is competitive, whereas inhibition by D-glucose is of the noncompetitive type. A number of trehalases have been found to be inhibited by sucrose. Thus, silkworm trehalase is competitively inhibited by sucrose (K_i 1.8 mM) and also inhibited by 2-amino-2-deoxy-D-glucose.¹³⁶ Sucrose also inhibits the trehalase of cockroaches,¹³⁸ ants,¹⁴¹ and honeybees.¹⁴² The significance of these inhibitions in terms of the metabolism of trehalose is not clear at this time.

As shown in Table II, trehalase has also been found in a number of mammalian species. Usually, the enzyme is localized in the microvilli of the intestine. This is not surprising, as most mammals undoubtedly ingest fairly large amounts of trehalose present in mushrooms, insects, or other organisms that store trehalose. However, trehalase activity has also been found in the renal, cortical tubules of a number of different mammals,¹⁵² as well as in the urine¹⁵⁴ and serum of humans.^{155,156} However, trehalase does not appear to be present in rat serum or, at most, is present in very low levels. Weser, Flores, and Young¹⁵⁸ injected [¹⁴C]trehalose into animals, and found very little carbon-14 in the carbon dioxide expired (5–6% of the total). On the other hand, when [¹⁴C]maltose or [¹⁴C]-malto-oligosaccharides were injected intravenously, 60–65% of the radioactivity was released¹⁵⁹ as [¹⁴C]carbon dioxide.

b. Activity in Developing Systems.—The metabolism of trehalose during development in various organisms has been studied in some detail, as this sugar is apparently an important energy-reserve. Hill and Sussman¹⁶⁰ examined the levels of the enzymes invertase and trehalase during the development of ascospores of *Neurospora crassa*. The highest trehalase activity occurred in ungerminated conidia, and the lowest in the ascospores. Although the specific activity of the trehalase varied no more than 3-fold during the spore stages, there was a 60-fold change in the mycelium. Sussman and Lingappa²¹ had previously shown that large amounts of trehalose are stored in dormant cells and that this trehalose is utilized during germination. Thus, the cleavage of trehalose is correlated with the breaking of dormancy and the activation of metabolism in ascospores. In terms of invertase and trehalase activity in *Neurospora*, Metzenberg¹⁶¹ found that growth of *Neurospora* on D-mannose-contain-

(158) E. Weser, personal communication.

(159) E. Weser, M. Friedman, and M. H. Sleisenger, *Biochim. Biophys. Acta.*, **136**, 170 (1967).

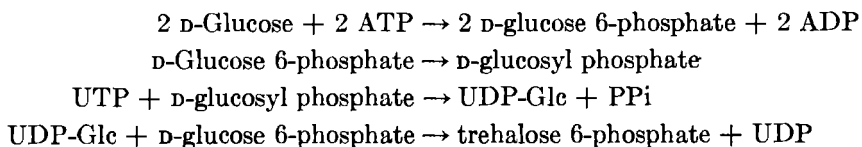
(160) E. P. Hill and A. S. Sussman, *J. Bacteriol.*, **88**, 1556 (1964).

(161) R. L. Metzenberg, *Arch. Biochem. Biophys.*, **96**, 468 (1962).

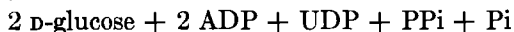
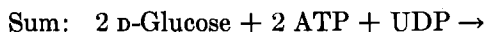
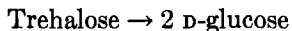
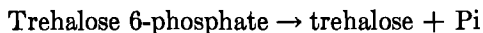
ing media resulted in low activities of invertase and trehalase, whereas very high levels of these enzymes were obtained during growth in a D-galactose-containing medium; he postulated that invertase and trehalase are coordinately repressed. However, the results of experiments by Hill and Sussman¹⁶⁰ did not support this hypothesis. Horikoshi and Ikeda¹²⁵ found that the content of trehalose in the conidia decreases at an early stage of germination, and postulated that trehalase might begin to hydrolyze trehalose after the inhibitory effect of D-mannitol has decreased.

Ceccarini¹⁶² studied the levels of trehalase during the life cycle of the cellular slime-mold *Dictyostelium discoideum*. The enzyme was found at the amebal stage, but, at aggregation, it was preferentially released and could be recovered in the external medium. Once the enzyme was released, the cells were only capable of synthesizing it *de novo* on feeding on *Escherichia coli*. Upon spore germination, trehalase reappeared, and trehalose was hydrolyzed to D-glucose. Ceccarini¹⁶² suggested that breakdown of the trehalose was necessary to spore germination. Some results analogous to those just described were obtained⁸³ with germinating spores of the actinomycete *Streptomyces hygroscopicus*. Spores of this organism were found to contain very low levels of the enzyme trehalase, but to have high concentrations of trehalose. Early in spore germination, before any increase in cell mass could be detected, the activity of trehalase began to increase, and it reached a maximum in ~25 hours, at which time its activity was about 20 times that in the original spores. During this same time-period, the levels of intracellular trehalose fell markedly. These results indicated that the enzyme trehalase may play an important role in the early events of spore germination.

c. Possible Role in Transport.—The discovery of a membrane-bound trehalase in the tubules of the renal cortex, as well as the possible presence of other enzymes involved in the biosynthesis of trehalose from D-glucose, led Sacktor¹⁵² to hypothesize that trehalose functions in the mechanism of resorption of D-glucose in the kidney. He proposed the following series of reactions to account for this hypothesis.



(162) C. Ceccarini, *Biochim. Biophys. Acta*, **148**, 114 (1967).



A similar role has been proposed¹⁵² for the trehalase found in intestine. However, Semenza and Rihova¹⁶³ found that, although 4',6'-dihydroxy-2'- β -D-glucopyranosyloxy-3-(*p*-hydroxyphenyl)propionophenone (phlorizin) inhibits the trehalase from both the small intestine and the kidney, the K_i of inhibition (2 mM) of the enzyme is some three orders of magnitude larger than the K_i of phlorizin inhibition of sugar transport in the small intestine (about 1 μ M). These data, as well as those from other studies, make unlikely the possibility that trehalase is involved in sugar transport in intestine; instead, it seems probable that this enzyme functions to degrade ingested trehalose.

3. α,α -Trehalose Phosphorylase

An enzyme that demonstrates a new pathway of trehalose catabolism has been found in *Euglena gracilis* by Marechal and Belocopitow.¹⁶⁴ This enzyme, which was purified 75-fold, catalyzes the reversible phosphorolytic cleavage of trehalose, yielding β -D-glucosyl phosphate and D-glucose as products. The optimal pH for phosphorolysis is 7.0, and, for synthesis, 6.3. The presence of such an enzyme in insects had been suggested in 1941 by Frèrejacque¹⁶⁵ as a result of experiments showing that degradation of trehalose in crude extracts is stimulated by phosphate; however, the presence of this phosphorylase in insects had not been confirmed. With the use of this phosphorylase, the authors¹⁶⁶ were able to prepare several hitherto-unknown disaccharides, notably 6-deoxy- α -D-glucopyranosyl α -D-glucopyranoside and α -D-xylopyranosyl α -D-glucopyranoside.

VI. SUMMARY OF THE ROLE OF α,α -TREHALOSE IN METABOLISM

1. As a Structural Component

In *Mycobacteria*, trehalose occurs not only as the free sugar but also as a component of various glycolipids, such as trehalose 6,6'-dimycolate.

(163) G. Semenza and L. Rihova, *Biochim. Biophys. Acta*, **171**, 393 (1969).

(164) L. R. Marechal and E. Belocopitow, *J. Biol. Chem.*, **247**, 3223 (1972).

(165) M. Frèrejacque, *Compt. Rend.*, **213**, 88 (1941).

(166) E. Belocopitow, L. R. Marechal, and E. G. Gros, *Carbohydr. Res.*, **19**, 268 (1971).

In order to determine the role of trehalose in these organisms, Elbein and Mitchell³⁷ examined the levels of free and "bound" (that is, lipid-associated) trehalose and glycogen during growth of *Mycobacterium smegmatis* under various conditions of nitrogen limitation. Whereas the glycogen levels increased markedly as the nitrogen content of the medium was lowered, the levels of trehalose remained fairly constant. The results of labeling studies suggested that the free trehalose in these cells may be utilized for purposes other than as an energy reserve, whereas glycogen is probably stored mainly as a reserve.

Winder and Brennan³⁸ found that *M. smegmatis* accumulated increased amounts of free trehalose shortly after exposure of the cells to isoniazid (isonicotinic acid hydrazide). This accumulation of trehalose appeared to be due to blocking by isoniazid of the synthesis of mycolic acid, and presumably, prevention of the incorporation of trehalose into trehalose 6,6'-dimycolate.¹⁶⁷ Winder and coworkers¹⁶⁸ found that, when *M. smegmatis* was in an approximately balanced state of growth on D-glucose as the sole carbon source, free trehalose turned over at about 3 times the rate of its net formation, while the sugars of O-acyltrehalose and O-acyl-D-glucose turned over at 13 and 8 times the rate of their net formation. Although these studies cast some doubt on the role of free trehalose as a precursor of O-acyltrehalose, it is possible that mycobacteria maintain separate pools of trehalose, or that the immediate precursor of O-acyltrehalose is trehalose 6-phosphate. At any rate, it seems probable that much of the trehalose synthesized by mycobacteria is destined to be used for structural purposes.

2. As a Reserve Supply of Energy

Studies, already discussed, dealing with development in fungi and streptomycetes have shown fairly conclusively that, during the initial stages of spore germination, trehalose is utilized as an energy source. These studies also established that the specific activity of the enzyme trehalase increases dramatically during (or just prior to) the time when trehalose is metabolized, making it likely that this enzyme is responsible for the initiation of catabolism of this sugar.

In insects, various workers have shown changes in the level of trehalose under various conditions. In 1955, Sacktor¹⁶⁹ showed that trehalose could support the respiration of housefly-muscle homogenates as readily as D-glucose or other substrates. Clegg and Evans⁶³ found that trehalose

(167) F. G. Winder and P. B. Collins, *J. Gen. Microbiol.*, **63**, 41 (1970).

(168) F. G. Winder, J. J. Tighe, and P. J. Brennan, *J. Gen. Microbiol.*, **73**, 539 (1972).

(169) B. Sacktor, *J. Biophys. Biochem. Cytol.*, **1**, 1 (1955).

provides the major source of flight energy in the adult blowfly (*Phormia regina*). During prolonged flight, the blood-trehalose level declined greatly, and the wing-beat frequency declined in synchrony with the trehalose, whereas the blood D-glucose levels remained fairly constant. The utilization of trehalose for flight has also been demonstrated in *Locusta migratoria*¹⁷⁰ and in the cockroach *Periplaneta americana*.⁴⁴

From the findings that the levels of blood trehalose fall during flight but are maintained during rest, and that the utilization of trehalose depends on the activity of trehalase, it seemed apparent that the activity of trehalase is regulated in relation to flight-muscle activity. This supposition was substantiated by Sacktor and Wormser-Shavit,¹⁷¹ who examined the levels of various substrates in the thoraces of *Phormia regina* taken after flights of different durations. The level of trehalose fell rapidly during the first minute, and then at a lower, but steady, rate, whereas that of D-glucose rose initially (presumably as a result of breakdown of trehalose), then fell, and finally regained a steady state. The mechanism of trehalase activation in these organisms, however, remains unknown. It was suggested by Wyatt¹⁷² that the very high concentration of trehalose in insect blood can be considered a direct and essential adaptation to flight.

3. As an Intermediate in D-Glucose Resorption or Mobility

As indicated previously (see p. 253), it has been suggested that synthesis and degradation of trehalose constitute a mechanism for the resorption of D-glucose in the kidney and, perhaps, also in the intestine. It has also been suggested that trehalose, like another naturally occurring, nonreducing disaccharide, sucrose, could function in the movement of carbohydrate, that is, as a translocate in plants or insects, or both.¹⁷³

There are other, as yet unexplained, "anomalies" in the biochemistry of trehalose, such as the presence of trehalase in human serum or human urine; this is thought to be due to release of renal trehalase. Another problem is the presence of trehalase in insect intestine, although trehalose is rarely an appreciable component of the insect diet; here, also, it has been speculated that trehalase is involved in the intestinal resorption of trehalose that has diffused from the hemolymph, through the gut wall, into the lumen.¹⁷³ Obviously, more experimentation to ascertain the role of trehalose and its relation to trehalase activity is necessary before the function in these organisms can be understood.

(170) T. Bucher and M. Klingenberg, *Angew. Chem.*, **70**, 552 (1958).

(171) B. Sacktor and W. Wormser-Shavit, *J. Biol. Chem.*, **241**, 624 (1966).

(172) G. R. Wyatt, *Advan. Insect Physiol.*, **4**, 287 (1967).

(173) W. N. Arnold, *J. Theoret. Biol.*, **21**, 13 (1968).

APPLICATION OF ENZYMIC METHODS TO THE STRUCTURAL ANALYSIS OF POLYSACCHARIDES*: PART I

BY J. JOHN MARSHALL

*Department of Biochemistry, University of Miami School of Medicine,
Miami, Florida 33152*

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I. INTRODUCTION

The classical techniques of structural analysis of polysaccharides, namely, fragmentation analysis (for example, by acid hydrolysis), methylation analysis, and periodate oxidation (for reviews, see Refs. 1-3) have

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been supplemented to an ever-increasing extent by enzymic methods. The use of enzymes in the analysis of polysaccharide structures has become so extensive that it is now appropriate to survey the progress made by the application of such methods. Previous articles^{4,5} have dealt with some of the earlier uses of enzymes in polysaccharide chemistry.

Early attempts to obtain structural information by using polysaccharide-degrading enzymes were, in many instances, regarded with suspicion by carbohydrate chemists, mainly because of their reservations that artifacts might arise through enzyme-catalyzed synthesis. However, now that we know more about these enzymes, particularly their specificities and mechanisms of action, and realize that polysaccharide biosynthesis does not occur by the direct reversal of hydrolytic enzyme action,^{5a} such suspicions have largely disappeared. In addition, the availability of improved methods for obtaining pure enzymes, often crystalline, means less likelihood of artifacts caused by the presence of contaminating enzymes, so that increased confidence may be placed in the results obtained.

Few exponents of the art of enzymic analysis of polysaccharide structure would claim that it replaces the classical, nonenzymic methods. One of the purposes of this article is to illustrate how the two types of method, enzymic and nonenzymic, may usefully complement each other. This result may be achieved in several ways. In its simplest form, it merely involves nonenzymic characterization of products of enzymic hydrolysis or, more commonly, characterization, by simple enzymic procedures, of products of low molecular weight that have been obtained by nonenzymic methods. In their most sophisticated form, enzymic methods alone are used for analysis of fine structure after a nonenzymic determination of the gross structural features of a polysaccharide has been made. Another variation is the consecutive use of enzymic and nonenzymic treatments at the polysaccharide level; for example, enzymic degradation after periodate oxidation, or after mild hydrolysis with acid to remove labile residues. Examples of all of these types of application will be considered. Contrary to some misconceptions, enzymic analysis does not result solely in qualitative information; instead, the most important uses of enzymes

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- (1) H. O. Bouveng and B. Lindberg, *Advan. Carbohydr. Chem.*, **15**, 53 (1960).
 - (2) E. L. Hirst, *Proc. Roy. Soc. A*, **252**, 287 (1959).
 - (3) E. G. V. Percival, "Structural Carbohydrate Chemistry," J. Garnet Miller, London, 2nd Edition, 1962.
 - (4) D. J. Manners, *Quart. Rev. (London)*, **9**, 73 (1955).
 - (5) D. J. Manners, *Roy. Inst. Chem. (London)*, *Lectures, Monographs, Repts.*, No. 2 (1959).
 - (5a) H. Nikaido and W. Z. Hassid, *Advan. Carbohydr. Chem. Biochem.*, **26**, 351 (1971).

are those that provide quantitative data. Frequently, quantitative information can be obtained more rapidly, and often more accurately, by enzymic than by nonenzymic methods. In many instances, the use of enzymes provides quantitative data that cannot be obtained by any nonenzymic method.

Besides application to the analysis of polysaccharides of unknown structure, enzymes are also often used for routine measurements on polysaccharides of essentially established structure, such as the starch components and the glycogens. Enzymes provide extremely quick and accurate information for the characterization of polysaccharides of this type, including the products synthesized *in vitro* during studies on their mechanism of biosynthesis. There is also increasing application to the characterization of other types of polysaccharide, synthesized enzymically *in vitro*. A further important use is for investigation of the nature of the products formed by the action of other catabolic enzymes on polysaccharides; such applications may give information on either the structure of the polysaccharide or the specificity of the catabolic enzyme under investigation.

For the enzymic analysis of polysaccharide structure, the hydrolytic enzymes are the most useful, and little attention will be paid in this article to other types of enzyme. No consideration at all will be accorded enzymes acting on non-carbohydrate moieties. For this reason, heteropolysaccharide-peptide and lipopolysaccharide complexes (such as those from bacterial cell-walls) are not discussed, as satisfactory treatment of these would also require mention of other types of enzyme, such as amidases; for coverage of this subject, an authoritative text⁶ and several reviews⁷⁻¹⁰ may be consulted. Other types of carbohydrate-containing macromolecule, such as glycosaminoglycans ("mucopolysaccharides") and glycoproteins, for which it is relatively easy to restrict consideration to the polysaccharide portion, are discussed, but only with reference to their carbohydrate moieties.

It was considered convenient to subdivide this article into Sections, each dealing with a particular class of polysaccharide, instead of into Sections dealing with different enzymes, the rationale being that the study of many polysaccharides has required several enzymes that, in some instances, are not involved in the breakdown of the polysaccharides

(6) M. R. J. Salton, "The Bacterial Cell Wall," Elsevier, Amsterdam, 1964, p. 133.

(7) J. L. Strominger and J. M. Ghuyssen, *Science*, **156**, 213 (1967).

(8) J. M. Ghuyssen, *Bacteriol. Rev.*, **32**, 425 (1968).

(9) J. M. Ghuyssen, J. L. Strominger, and D. J. Tipper, in "Comprehensive Biochemistry," M. Florkin and E. H. Stotz, eds., Elsevier, Amsterdam, 1968, Vol. 26A, p. 53.

(10) M. J. Osborn, *Ann. Rev. Biochem.*, **38**, 501 (1969).

in vivo. As certain enzymes have been used in structural studies on polysaccharides of different types, it should not be surprising to find some enzymes mentioned in several Sections. In each Section, the most important enzymes that have been applied, or are of potential application, to the particular class of polysaccharide under discussion, are described insofar as availability, purity, specificity, and other relevant properties are concerned. No consideration has been given to the assay of activity of these enzymes; this will be discussed in detail elsewhere.¹¹ Mention of the relevant enzymes is then followed by a discussion of their applications. No attempt has been made to provide an exhaustive compilation of all such uses. Instead, the ways in which enzymes may be utilized, and the advantages associated with their use, have been illustrated by some of the most important applications. In certain cases, the opportunity has been taken to speculate on potential uses of enzymes in structural work, in addition to applications already reported. Thus, possible uses both of existing and of thus far unknown enzymes have been suggested.

No attempt will be made to overstress the merits of enzymic methods in the analysis of polysaccharide structure. Because important lessons may be learned from the unsuccessful, or even careless, use of enzymes, examples of such applications are given. Awareness of the problems and shortcomings associated with the use of enzymes in structural analysis can only be to the advantage of any potential user, and these have, therefore, been outlined, particularly such aspects as the problems associated with the use of impure or inadequately characterized enzymes.

One group of polysaccharides, namely, the amylaceous type, has been considered most extensively (see Section IV, p. 285). This group provides some of the most sophisticated examples of how the application of highly purified enzymes, having rigorously ascertained specificities, may be used to obtain information that no nonenzymic method could ever provide.¹² Furthermore, the studies on the structures of the products formed by the enzymic degradation of these polysaccharides *in vitro* illustrate how information may be obtained on the mechanism of their catabolism *in vivo*.

II. PREPARATION, CHARACTERIZATION, AND SOME ASPECTS OF THE USE OF ENZYMES

In order to utilize enzymic methods in structural analysis, it is necessary to understand the basic principles of enzymology, particularly en-

(11) J. J. Marshall, to be published.

(12) W. J. Whelan, *Biochem. J.*, **122**, 609 (1971).

zyme kinetics and the techniques of protein purification. Enzyme kinetics are discussed in all books on general biochemistry (see, for example, Refs. 13 and 14); in addition, more-comprehensive works are also available.^{15,15a,16} Purification of proteins has been discussed in detail in several texts.¹⁷⁻¹⁹ This Section will be restricted mainly to the most important practical aspects of the use of enzymes.

Because all of the conclusions reached by the application of enzymes depend on a knowledge of the specificity and action pattern of the particular enzyme being used, the importance of obtaining enzymes that are homogeneous and properly characterized must be realized. Attention paid to the points to be discussed should aid in the achievement of satisfactory results and help in avoiding wrong conclusions.

1. Preparation of Enzymes

a. Sources.—Where such well characterized, commercially available enzymes as *beta*-amylase are to be used, the most important points to be considered in selecting the source of the enzyme are the standards of purity and prices of the enzymes from different suppliers.

On the other hand, where the enzyme required is not commercially available, choice of the source of the enzyme may be an important consideration. Such factors as the ease of obtaining the starting material and the differences in specificity of the "same" enzyme from different origins must be taken into account, and that chosen which gives an enzyme having the most suitable specificity. In addition, the ease of purification of the enzyme from different starting materials may differ, and one for which an established purification procedure is available is to be preferred. Generally, the enzyme requiring the smallest number of purification steps should be chosen. Sources in which the enzyme that is

(13) A. L. Lehninger, "Biochemistry," Worth, New York, 1970.

(14) A. White, P. Handler, and E. L. Smith, "Principles of Biochemistry," McGraw-Hill, New York, 5th Edition, 1973.

(15) K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford Univ. Press, Oxford, 1958.

(15a) J. M. Reiner, "Behavior of Enzyme Systems," Van Nostrand-Reinhold, New York, 2nd Edition, 1969.

(16) P. D. Boyer, ed., "The Enzymes," Academic Press, New York, 3rd Edition, 1970, Vol. 2. In subsequent References, this series is denoted as *Enzymes*.

(17) J. H. Norris and D. W. Ribbons, eds., "Methods in Microbiology," Academic Press, London, 1971, Vol. 5B.

(18) W. B. Jakoby, ed., "Methods in Enzymology," Academic Press, New York, 1971, Vol. 22.

(19) J. J. Marshall, "An Introduction to Protein Purification," Koch-Light Laboratories Ltd., Colnbrook, Bucks., England, 1972.

needed coexists with related enzymes, difficult to remove, that might interfere with the proposed use of the enzyme, are to be avoided as far as possible.

Sometimes, the enzyme that would be the most useful may not even be known; it may then be necessary to screen a large number of plants, animal tissues, and micro-organisms, using, where possible, a specific substrate for the enzyme, until one is found where the enzyme is present constitutively in sufficient proportion to make extraction and purification feasible.

b. Enzyme Induction.—Where a suitable enzyme is either not known, or present only as a minor component of a mixture from which purification is difficult, resort is frequently made to the technique of enzyme induction in micro-organisms to obtain the enzyme. The principle of the method is that growth of a suitable organism on a selected, carbon source may be used to give an enzyme previously absent, or to increase the level of an enzyme previously present in only small proportion. Most commonly, there is a high degree of structural similarity between the inducer and the substrate of the enzyme required; often, the inducing carbon source is the polysaccharide for which degradative enzymes are being sought. Examples are the induction of *exo*-mannanase by growth of a species of *Arthrobacter* on yeast mannan,²⁰ of mycodextranase by growth of *Penicillium melinii* on nigeran,²¹ and of inulinase by a species of *Penicillium* growing on inulin.²² Pullulanase is induced in *Aerobacter aerogenes* by maltose, soluble starch, or pullulan.^{23,24} Modified substrates of the desired enzyme may also be useful, for example, palmitic esters of isomaltose for the induction of dextranase.²⁵ This procedure is particularly useful where the inducer (isomaltose for dextranase) acts also as a repressor; the repression is avoided by slowly supplying the disaccharide, which is formed by the action of induced esterases on the palmitic esters. Thus, growth of *Trichoderma viride* on isomaltose dipalmitate gave yields of dextranase ~700 times those obtained by growth on the unsubstituted disaccharide.²⁵

Another type of technique that may be employed takes advantage of continuous culture in a chemostat to provide extremely high levels of a desired enzyme. Conditions that have been used include growth on limiting concentration-levels of the substrate of the desired enzyme, and

(20) G. H. Jones and C. E. Ballou, *J. Biol. Chem.*, **244**, 1043 (1969).

(21) E. T. Reese and M. Mandels, *Can. J. Microbiol.*, **10**, 103 (1964).

(22) T. Nakamura and S. Hoashi, *Nippon Nogei Kagaku Kaishi*, **43**, 599 (1969).

(23) Y. Fujio, M. Sambuichi, and S. Ueda, *Hakko Kogaku Zasshi*, **48**, 601 (1970).

(24) C. Mercier, B. M. Frantz, and W. J. Whelan, *Eur. J. Biochem.*, **26**, 1 (1972).

(25) E. T. Reese, J. E. Lola, and F. W. Parrish, *J. Bacteriol.*, **100**, 1151 (1969).

growth on a poor substrate of the enzyme as carbon source for the organism. Thus, the β -D-galactosidase of *Escherichia coli* has been produced in large amounts by growth on very low levels of lactose.²⁶ Ribitol dehydrogenase may be obtained from a mutant of *Aerobacter aerogenes* by growth on xylitol;²⁷ this organism has a constituent ribitol dehydrogenase,²⁸ an enzyme for which xylitol is a very poor but utilizable substrate. In both of these instances, growth under the conditions described resulted in a large increase, after several generation times, in the amounts of the enzymes (β -D-galactosidase and ribitol dehydrogenase) synthesized, presumably as a result of gene doubling. In both instances, the enzyme protein constituted a relatively high percentage of the total protein present, so that purification was facilitated. It should be fairly straightforward to extend this method and to develop similar conditions for production of large amounts of other useful enzymes.

One of the simplest ways of finding organisms producing enzymes capable of degrading specific substrates is by enrichment culture, involving growth of a mixture of micro-organisms, using as the sole carbon source the substrate for which the degradative enzymes are required. Usually, the micro-organisms are from such natural sources as soil and decaying vegetation. After initial growth of the organisms in a sample of the starting material, followed by repeated, serial transfers and isolation of individual colonies by streaking on plates containing the substrate as sole carbon source, it is usually possible to isolate pure cultures of one or more organisms producing enzymes that degrade it. Clearly, organisms producing such enzymes constitutively, as well as by induction by substrate, will be obtained. Cultivation of the organisms can then be used to give crude enzyme preparations from which the enzymes involved in degradation of the substrate may be purified and characterized.

These methods have been mentioned because it is probable that micro-organisms will become increasingly important as sources of enzymes for structural analysis; this is likely in view of the wide variety of micro-organisms available and of an ever-increasing understanding of their biochemistry and genetics. In particular, it is likely that induction methods will become one of the best ways to obtain enzymes capable of degrading such complex polysaccharides as the plant gums, studies on which have, until now, been restricted to the use of purely nonenzymic methods alone. A potentially useful technique for determining the structures of

(26) C. A. Novick and T. Horiuchi, *Cold Spring Harbor Symp. Quant. Biol.*, **26**, 239 (1961).

(27) B. S. Hartley, *Abstr. 9th Int. Congr. Biochem.*, Stockholm, 7 (1973).

(28) E. C. C. Lin, in "Miami Winter Symposia," W. J. Whelan and J. Schultz, eds., North Holland, Amsterdam, 1970, Vol. 1, p. 89.

some of the more complex polysaccharides, based on the enzymes that they induce, is described in Part 3 of this Section (see p. 273).

c. Criteria of Purity.—The standard of purity of enzyme preparations used in structural work is very important. The use of impure preparations containing interfering activities generally leads to incorrect or ambiguous results, as will be seen later. For this reason, an evaluation of the degree of purity of any enzyme preparation is essential; this should not be restricted to enzymes prepared in the laboratory, but should also be applied to commercially available enzymes. Crystalline, commercial enzymes often contain contaminating activities (see, for example, Ref. 29). The purer the preparation used, the more confidence can be placed in the significance of the results obtained.

The two most common ways of estimating the degree of homogeneity of enzyme preparations are ultracentrifugation and electrophoretic analysis. A sharp boundary on sedimentation in the ultracentrifuge is often taken to indicate homogeneity. However, electrophoresis, particularly in poly(acrylamide) gels (so-called "disc-gel" electrophoresis³⁰), is a somewhat more sensitive procedure for detecting minor impurities; it has the added advantage that duplicate samples may be employed, one to be used for location of protein by a suitable staining-procedure³¹ and the other for location of enzymic activity. The latter procedure may be conducted in two ways; either by cutting out zones and incubating with substrate, or, if a suitable staining-method is available, by location *in situ*. Thus, for enzymes hydrolyzing polysaccharides, triphenyltetrazolium chloride is often used for locating the products of enzyme action (after electrophoresis, and incubation with substrate).³² In this way, it is possible to ascertain whether the major component is, indeed, the enzymically active protein desired.

The advantages associated with the use of rigorously purified enzyme-preparations cannot be overemphasized, and time spent in isolating an enzyme in homogeneous form is generally time well spent. However, some workers wishing to use enzymes as structural tools avoid exhaustive purification. Often, the standard of purity aimed for is restricted to functional purity, that is, the absence of interfering enzymic activities. This is achieved by purifying the desired enzyme until it has no action

(29) E. Y. C. Lee and W. J. Whelan, *Biochem. J.*, **95**, 27F (1965).

(30) L. Ornstein, *Ann. N. Y. Acad. Sci.*, **121**, 321 (1964); B. J. Davis, *ibid.*, **121**, 404 (1964).

(31) A. Chambach, R. A. Reisfeld, M. Wycoff, and J. Zaccari, *Anal. Biochem.*, **20**, 150 (1967).

(32) O. Gabriel and S.-F. Wang, *Anal. Biochem.*, **27**, 545 (1969).

on other substrates that would detect interfering enzymic activities. Thus, a starch- or glycogen-debranching enzyme-preparation would be functionally pure if it contained no other amylolytic enzymes or α -D-glycosyl transferases. In many instances, such preparations give satisfactory results, although ambiguities are more likely to arise than when homogeneous preparations are used. It is sometimes possible to obtain meaningful results by using enzyme preparations in which interfering activities have been selectively inhibited. Thus α - and β -D-glucosidases may be selectively inhibited in *exo*- and *endo*-glucanase preparations by using such polyhydric alcohols as 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), glycerol, or erythritol,^{32a} thioglycosides,^{32b,c} lactones,^{32d,e,f} or nojirimycin, an antibiotic substance differing only from D-glucose by the substitution of an NH grouping for the ring-oxygen atom.^{32f,g} Similarly, β -D-xylosidase may be selectively inhibited with D-xylose derivatives having sulfur or nitrogen in the ring.^{32h} In general, however, inhibition of contaminating activities in this way is less satisfactory than their complete removal.

One particularly difficult task has been the detection of *endo*-acting enzymes in preparations of *exo*-enzymes.³³ Enzymes of the latter type are particularly useful in structural analysis; for example, in the removal of all nonreducing end-residues in a polysaccharide susceptible to the action of the enzyme. When chains of susceptible residues are present, these are degraded by the endwise action of the enzyme until a non-susceptible linkage or residue is encountered. In either instance, measurement of the degree of hydrolysis may give useful results. However, the presence of an *endo*-enzyme is likely to make such measurements meaningless, as its lack of requirement for a chain end may render some residues susceptible to action of *exo*-enzyme, for example, residues in the interior chains or backbone of a polysaccharide, which otherwise would not have been attacked. Even traces of *endo*-enzyme are sufficient to create difficulties,

(32a) M. V. Kelemen and W. J. Whelan, *Arch. Biochem. Biophys.*, **117**, 423 (1966).

(32b) H. Halvorson and E. Ellias, *Biochim. Biophys. Acta*, **30**, 28 (1958).

(32c) K. Wallenfels, J. Lehmann, and O. P. Malhotra, *Biochem. Z.*, **332**, 209 (1960).

(32d) J. Conchie, A. L. Gelman, and G. A. Levvy, *Biochem. J.*, **103**, 609 (1968).

(32e) G. A. Levvy, A. J. Hay, and J. Conchie, *Biochem. J.*, **91**, 378 (1964).

(32f) E. T. Reese, F. W. Parrish, and M. Ettlinger, *Carbohydr. Res.*, **18**, 381 (1971).

(32g) T. Niwa, S. Inouye, T. Tsuruoka, Y. Koaze, and T. Niida, *Agr. Biol. Chem. (Tokyo)*, **34**, 966 (1970).

(32h) M. Claeysens and C. K. De Bruyne, *Naturwissenschaften*, **52**, 515 (1965).

(33) Enzymes having *exo* activity remove units successively from nonreducing chain-ends; *endo*-acting enzymes degrade polysaccharide chains in a more random manner (see also, p. 270).

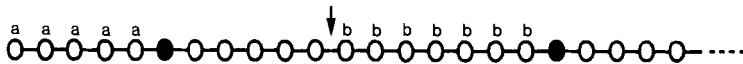


FIG. 1.—Diagrammatic Representation of a Segment Near the Nonreducing End of an Amylose Chain in which a Small Proportion of the D-Glucose Residues Have Been Oxidized with Sodium Metaperiodate. [○ represents a D-glucose residue; ●, an oxidized D-glucose residue; and —, a (1→4)- α -D-glucosidic linkage. Action of glucoamylase results in release of the D-glucose residues (a) to the nonreducing (“left”) side of the first oxidation point, but the presence of contaminating *alpha*-amylase results in *endo* cleavages (for example, at the point indicated by the arrow), with liberation of new, nonreducing chain-ends, allowing D-glucose residues (b) between oxidation points to be liberated by enzyme action.]

and such small amounts may be extremely difficult to detect. An example is the detection of *alpha*-amylase in glucoamylase preparations, where the latter enzyme will act on any nonreducing chain-ends liberated by *alpha*-amylase, so that the only product observed is D-glucose.

A procedure has been developed for the detection of such impurities,^{34,35} the method being illustrated by consideration of the glucoamylase-*alpha*-amylase situation just mentioned. This involves preparation of a substrate (see Fig. 1) containing blockages to *exo*-enzyme action, achieved by a limited ($\sim 5\%$) oxidation of amylose with sodium metaperiodate. Comparison of the action on this substrate of glucoamylase with and without *alpha*-amylase contamination is shown in Fig. 2. Action of crystalline glucoamylase ceased after 4–5% of hydrolysis, corresponding to removal of all of the D-glucose residues from the nonreducing end to

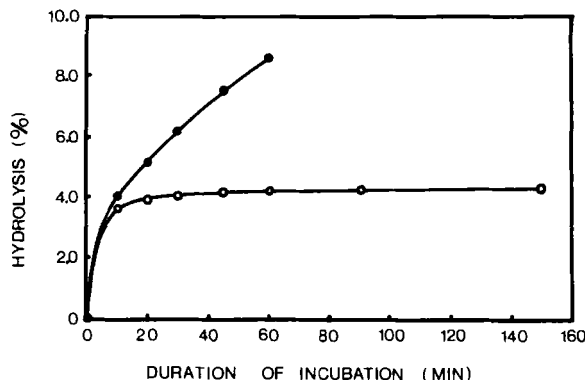


FIG. 2.—Degradation³⁵ of Oxidized Amylose (see Fig. 1) by Pure Glucoamylase (○) and by Glucoamylase Contaminated with *alpha*-Amylase (●).

(34) E. E. Smith, G. S. Drummond, J. J. Marshall, and W. J. Whelan, *Fed. Proc.*, **29**, 930 (1970).

(35) J. J. Marshall and W. J. Whelan, *Anal. Biochem.*, **43**, 316 (1971).

the first oxidation point in each chain. The presence of *alpha*-amylase in amorphous glucoamylase was detected by a hydrolysis beyond this extent, as a result of other D-glucose residues, namely, those between oxidation points, being rendered susceptible to glucoamylase action by the *endo* action of *alpha*-amylase. This is a completely general method, applicable to any polysaccharide and the appropriate *exo*-enzyme. In its original form,³⁶ it was applied to the laminaran-*exo*-(1→3)-β-D-glucanase system, where the enzyme action was prevented by oxidation of the non-reducing end-group in the chain, the only residue (other than the reducing-end residue) oxidized by periodate. It was not realized at that time how generally applicable is the method. The same effect is obtained by using a chromogenic substrate in which the blockages to *exo*-enzyme action are the chromogenic groups randomly distributed on some of the D-glucose residues.³⁷

2. Characterization of Enzymes

a. **General Properties.**—Every enzyme has a range of optimum conditions for activity; these should either be found by reference to the literature, or established by a detailed examination of its properties. Although results may be obtained under non-optimal conditions, only when the conditions most favorable for enzyme action are adhered to are satisfactory and reproducible results generally obtained.

The factors influencing enzyme activity are discussed in such textbooks as those already mentioned,^{13,14} but may be summarized here. Briefly, use of any enzyme should be restricted to a temperature below that causing inactivation. The pH at which the enzyme is used should be such that the enzyme is both active and stable; this can only be ascertained by studying the pH-stability and pH-activity properties of the enzyme. The matter of stability is particularly important when incubations are to be prolonged. Care must be taken when enzymes having alkaline pH requirements are used, because, under such conditions, alkali-catalyzed epimerization of monosaccharide residues may take place, particularly if heat treatment is employed for enzyme inactivation.

Other properties usually investigated are the requirements for cofactors to activate, or stabilize, the enzyme. This information then allows use of the enzyme under conditions where there are unlikely to be any problems arising from loss of activity. Thus, calcium ions should be present in *alpha*-amylase digests in order to stabilize the enzyme,

(36) T. E. Nelson, J. V. Scaletti, F. Smith, and S. Kirkwood, *Can. J. Chem.*, **41**, 1671 (1963).

(37) J. J. Marshall, *Anal. Biochem.*, **37**, 466 (1970).

particularly if any protease is present;³⁸ in a similar way, it is required for β -D-glucan hydrolase activity to the extent that some such enzymes are inactive in the absence of this cation.³⁹

The possibility of inhibition of an enzyme by its product is another item to be considered. If this phenomenon is observed, the enzyme must always be used under conditions where the concentration of product does not exceed that causing inhibition.

A special situation arises where two enzymes are used simultaneously. In such instances, consideration of the properties of both enzymes can be used to select conditions where both are reasonably active, although often not optimally so. This is particularly important when coupled enzymes are used for analytical measurements.

b. Specificity.—Before an enzyme can be of use in the elucidation of polysaccharide structure, its specificity must be ascertained. This is done by testing both the extent and rate of action of the enzyme on a series of model compounds of defined structure. After the specificity has been established, the enzyme may be used for analysis of a polysaccharide of unknown structure. Examples will frequently be encountered where enzymes of unknown specificity have been used to degrade polysaccharides of unknown structure. Such experiments are usually pointless, because, as has been emphasized by French,⁴⁰ the same experiment cannot simultaneously reveal both the structure of the substrate and the action and specificity of the enzyme.

There are three specificity requirements to be ascertained for the glycoside hydrolases: specificity with respect to (i) the type of linkage hydrolyzed, (ii) the glycon, and (iii) the aglycon. For the simple glycosidases, these requirements may all be readily determined by using a selection of glycosides. In the case of enzymes acting on polysaccharides, this procedure may be rather more difficult, particularly in view of the dearth of suitable model compounds; usually, however, these are obtained from a relatively complex substrate by degradation with an acid or an enzyme, and then characterized. For example, the specificities of two (1 \rightarrow 6)- α -D-glycosidases, pullulanase⁴¹ and isoamylase,^{42,43} have been

(38) E. H. Fischer and E. A. Stein, *Enzymes*, 4, 313 (1960).

(39) J. J. Marshall, to be published.

(40) D. French, *Enzymes*, 4, 345 (1960).

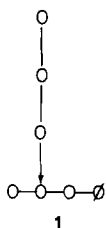
(41) M. Abdullah, B. J. Catley, E. Y. C. Lee, J. Robyt, K. Wallenfels, and W. J. Whelan, *Cereal Chem.*, 43, 111 (1966).

(42) Z. Gunja-Smith, J. J. Marshall, E. E. Smith, and W. J. Whelan, *FEBS Lett.*, 12, 96 (1970).

(43) J. J. Marshall, Z. Gunja-Smith, and E. E. Smith, *Fed. Proc.*, 30, 1064 (1971).

determined by using a series of degradation products having known structures, produced by the action of other enzymes on glycogen and amylopectin.

Some enzymes show a relatively high specificity requirement, whereas that of others is considerably lower. Thus, the enzyme laminaranase from *Rhizopus arrhizus*⁴⁴ has a specificity requirement for only one linkage in the substrate, which must necessarily be (1→3)-β-D in nature. The low specificity of the enzyme allows it to cleave^{45-47a} either (1→3)-β-D- or (1→4)-β-D-linkages involving the D-glucose residue substituted at O-3. On the other hand, the enzyme isoamylase shows a high specificity requirement. Present knowledge of the specificity requirements of the latter enzyme shows that it covers at least four linkages, probably even more.^{43,48} The smallest substrate known at present is 6³-α-maltotriosyl-maltotetraose⁴⁹ (1), in which the (1→6)-α-D-glucosidic linkage and the



three (1→4)-α-D-glucosidic linkages on the nonreducing sides of the branch point are known to be involved in the specificity of the enzyme. The possibility that 6²-α-maltotriosylmaltotriose (2) or 6¹-α-maltotriosyl-maltose (3) are substrates cannot be excluded, so that the question of

(44) E. T. Reese and M. Mandels, *Can. J. Microbiol.*, **5**, 173 (1959).

(45) F. W. Parrish, A. S. Perlin, and E. T. Reese, *Can. J. Chem.*, **38**, 2094 (1960).

(46) F. W. Parrish and A. S. Perlin, *Nature*, **187**, 1110 (1960).

(47) A. S. Perlin and S. Suzuki, *Can. J. Chem.*, **40**, 50 (1962).

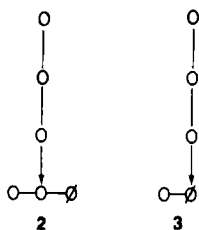
(47a) A. S. Perlin, in "Advances in Enzymic Hydrolysis of Cellulose and Related Materials," E. T. Reese, ed., Pergamon Press, New York, 1963, p. 185.

(48) Z. Gunja-Smith, J. J. Marshall, E. E. Smith, and W. J. Whelan, unpublished work (1971).

(49) In this article, for convenience, the nomenclature used for the starch-derived oligosaccharides is that introduced by Whelan,^{50,50a} and the residues are considered to be pyranoid. Such oligosaccharides are represented diagrammatically by using ○ for a (nonreducing) α-D-glucosyl residue, ∅ for a (reducing) D-glucose residue, — for a (1→4)-α-D-glucosidic linkage, and ↓ for a (1→6)-α-D-glucosidic linkage.

(50) W. J. Whelan, *Ann. Rev. Biochem.*, **29**, 105 (1960).

(50a) R. W. Bailey, "Oligosaccharides," Pergamon Press, Oxford, 1965, p. 3.



whether or not one, or both, of the other two α -glucosidic linkages [that is, those on the reducing ("right") side of the branch point in 1] are involved in the specificity of the enzyme can not as yet be answered.

The important point is that, the higher the specificity of an enzyme, the more useful the enzyme is likely to be in polysaccharide structural analysis. The amount of reliable information that can be obtained by the use of enzymes having a low degree of specificity is strictly limited. Such an example is provided by the enzyme β - α -glucosidase from almond emulsin. This enzyme shows such a low specificity for the linkage hydrolyzed and the glycon and aglycon portions of the substrate,^{52a,52d,51-53} that the only information that can be obtained from an observation of an increase in reducing power resulting from its action is that there are non-reducing-end, β - α -linked monosaccharide residues.⁵⁴ Whether these are (1 \rightarrow 1)-, (1 \rightarrow 2)-, (1 \rightarrow 3)-, (1 \rightarrow 4)-, or (1 \rightarrow 6)- β - α -glycosidic linkages, and the monosaccharide residues are those of glucose, galactose, or xylose, is not revealed without performing other experiments.

c. Action Pattern.—It is important that, at an early stage in the examination of an enzyme, the mechanism by which it degrades the substrate be determined. The mechanism may be either *exo* or *endo*. Enzymes that have *endo*-activity break down the polysaccharide into fragments by hydrolysis of all susceptible linkages in an essentially random fashion. Enzymes that have *exo*-activity degrade the polysaccharide in a stepwise manner, from the nonreducing chain-ends, by liberation of mono- or oligo-saccharide units until a structural feature is reached (for example, a point of branching) where the enzyme action is halted. There are several methods for determining whether the action pattern of an enzyme is *exo* or *endo*.

(51) H. Baumann and W. Pigman, in "The Carbohydrates," W. Pigman, ed., Academic Press, New York, 1957, p. 536.

(52) D. J. Manners and D. C. Taylor, *Carbohydr. Res.*, **7**, 497 (1968).

(53) J. Conchie, A. L. Gelman, and G. A. Levvy, *Biochem. J.*, **106**, 135 (1968).

(54) Or, possibly, even L-monosaccharides joined through α -L-linkages.

Measurement of changes in viscosity was one of the earliest methods for examining the action patterns of enzymes, the basis of the method being that *endo* (random) cleavage results in rapid loss of viscosity, whereas stepwise degradation gives only a slow loss of viscosity. Thus, there is a very large difference in the viscosity decrease at the same degree of hydrolysis by an *exo*- and an *endo*-enzyme.

Chromatographic fractionation after partial enzymic hydrolysis can distinguish between the two types of action pattern. Action that is *exo* will give only a single, chromatographically mobile product (except in the very late stages of reaction, where oligosaccharides from the non-degraded part of linear chains may be observed); *endo* action gives a series of products ranging in size from that of the smallest oligosaccharide produced by the enzyme to almost that of the starting material. In such a way, the action of pullulanase on pullulan was shown to be *endo*,⁵⁵ its action giving a polymer-homologous series consisting of (1→6)-linked α -maltotriosyl residues. This result was in contrast to that from an earlier study,⁵⁶ which had suggested, on the basis of g.l.c. evidence, that the enzyme acted on pullulan in an *exo* fashion, liberating maltotriose. The chromatographic method has the possible disadvantage that, should the intermediate products be more rapidly degraded than the starting material, the action will appear to be *exo* (not *endo*), as only the product of lowest molecular weight will be readily apparent. Sometimes, it is convenient to examine the products of enzyme action by chromatography on a molecular sieve (such as Sephadex or Biogel). Action of the *exo* type gives only two peaks, which, if the appropriate medium is used, are, respectively, excluded from the gel (incompletely degraded, macromolecular material) and retained by the gel, emerging as a single, sharp peak near the total volume of the column (liberated mono- or oligosaccharide). This column-chromatographic method becomes most convincing when the degree of polymerization (d.p.) of the non-excluded material is measured at different extents of hydrolysis of the substrate.⁵⁷ For an *exo*-enzyme, the d.p. remains the same at all extents of hydrolysis; when the action is *endo*, it falls continuously. It is, however, important to realize that misleading results may be obtained by application of this method to certain macromolecular substrates, such as the highly ramified polymers glycogen and amylopectin, where steric effects appear to force some *endo*-acting enzymes to act by an apparent *exo*-type of action. This

(55) G. S. Drummond, E. E. Smith, W. J. Whelan, and H. Tai, *FEBS Lett.*, **5**, 85 (1969).

(56) K. Wallenfels, I. R. Rached, and F. Hucho, *Eur. J. Biochem.*, **7**, 231 (1969).

(57) K. K. Tung and J. H. Nordin, *Anal. Biochem.*, **29**, 84 (1969).

behavior is observed in the degradation of these polysaccharides by debranching enzymes. That the action is not a true *exo* action is, however, apparent from the degradation of phosphorylase-*beta*-amylase (ϕ -*beta*) limit dextrins (see p. 315) of amylopectin and glycogen by isoamylase. In the initial stages of degradation of these substrates, the material liberated must be chains carrying maltosyl branches near the nonreducing end, afforded by an *endo* type of action.

Another method involves measurement of the rate of formation of the products of enzyme action by two different methods, this being most conveniently applied when D-glucose is liberated. In such a situation, D-glucose would be measured specifically by the D-glucose oxidase assay⁵⁸ and, in addition, the total amount of reducing sugar liberated would be measured (as D-glucose equivalents) by, for example, a reducing-power method.⁵⁹ When these two values are identical, this indicates an *exo* mechanism. When the amount of "glucose" measured by the reducing power is greater than that determined by the specific, enzymic assay, an *endo* mechanism is indicated.

Modified substrates may be used in testing the action patterns of purified enzymes. Thus, periodate-oxidized laminaran was used by Smith and coworkers to show the *exo* action of Basidiomycete (1 \rightarrow 3)- β -D-glucanase.⁵⁸ The oxidized-amylose substrate described earlier (see p. 266) has been used with *alpha*- and *beta*-amylases to illustrate how the method is completely general, and applicable to all classes of polysaccharides and polysaccharidases.⁶⁰ By use of oxidized pullulan, the *endo* nature of pullulanase action was confirmed.⁶⁰

3. Special Techniques

Four enzymic methods that have been of use in the analysis of polysaccharide structure are considered here.

a. **Isolation of Products of Partial Enzymic Depolymerization.**—Sometimes, it is desirable to obtain products formed by partial breakdown of the substrate, rather than the products of unrestricted enzyme action. One example of such a situation is that in which an enzyme preparation is used that is contaminated with small proportions of glycosidases acting on the products of action of the major enzyme.

A better method than enzyme inactivation after limited enzyme action,

(58) J. B. Lloyd and W. J. Whelan, *Anal. Biochem.*, **30**, 467 (1969).

(59) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944); M. Somogyi, *ibid.*, **195**, 19 (1952).

(60) G. S. Drummond, E. E. Smith, and W. J. Whelan, *FEBS Lett.*, **15**, 302 (1971).

particularly because the yields of intermediate products may be greatly increased, is to allow the products to be continuously removed from the site of enzyme action by passage through a membrane. A common procedure is to allow reaction to take place in a dialysis bag that retains the enzyme and macromolecular substrate, but allows the primary degradation products to escape.⁶¹

The development and commercial availability of apparatus for ultrafiltration, and of membranes of controlled porosity, has allowed an improvement in this method. The principle is illustrated by the continuous, enzymic degradation of amylaceous substrates,^{62,63} but it is applicable to the degradation of any polysaccharide by a suitable enzyme-preparation. This reaction takes place in an ultrafiltration cell under pressure, and the products emerge through a membrane at the bottom. One of the advantages of this method is that the molecular size of the products emerging may be controlled by altering the porosity of the membrane, although care has to be taken that it is suitably chosen to retain both the original substrate and the enzyme. In addition, there is no chance that the products of low molecular weight will diffuse back to the site of enzyme action; this may occur when the dialysis procedure is used without the passage of a continuous stream of liquid.

This ultrafiltration technique also constitutes an improved method for testing the *exo* or *endo* nature of the action of an enzyme. The principle is the same as that used by Tung and Nordin,⁵⁷ but the necessity of column-chromatographic separation of products and macromolecular material is eliminated. Thus, all that would be necessary would be estimation of the d.p. of the products in the ultrafiltrate after various times of reaction.

b. Sequential Induction of Enzymes.—Monod⁶⁴ has shown that utilization of the components of a sugar mixture by micro-organisms takes place in a sequential manner. The enzyme system needed to initiate the attack of a particular component is not produced until sugars preferentially utilized are exhausted. Similarly, when a complex substrate, such as a heteropolysaccharide, is presented to a suitable organism growing on a suitable carbon source (such as succinate), the enzymes required for degradation of the polysaccharide are produced sequentially. The

(61) T. J. Painter, *Can. J. Chem.*, **37**, 497 (1959).

(62) T. A. Butterworth, D. I. C. Wang, and A. J. Sinskey, *Biotechnol. Bioeng.*, **12**, 615 (1970).

(63) J. J. Marshall and W. J. Whelan, *Chem. Ind. (London)*, 701 (1971).

(64) J. Monod, "Recherches sur la Croissance des Cultures Bactériennes," Hermann et Cie, Paris, 1942.

nonreducing chain-ends act as inducers at each stage in the degradation. The enzyme first induced removes its inducing site, exposing a different sugar at the chain ends. These, in turn, act as inducing sites, and a second induced enzyme is then synthesized, and so on. Only one induced enzyme is active at any one time. The process is known as sequential, enzyme induction.^{65,66}

The products of enzyme action must not be utilized by the organism; this can be ensured by the presence of an excess of a carbon source such as succinate in the culture medium. The results of qualitative and quantitative analysis of the products released can then be used to deduce the arrangement of the sugar residues in the chain. A study of the specificities of the induced enzymes may lead to information on the configuration and mode of linkage of the sugar residues.

This technique has been useful for the examination of pneumococcal polysaccharides and the carbohydrate moieties of certain glycoproteins, by induction of enzymes produced by a strain of *Aerobacter aerogenes*.^{65,67-70}

c. Use of *exo*-Enzymes to Determine the Linear or Branched Nature of Polysaccharides.—Substrates oxidized to a limited extent with periodate [a procedure found useful for testing the purity and action patterns of glycoside hydrolases (see p. 266)] may also be employed to determine whether a polysaccharide is linear or branched.^{12,71} The rationale is that the same extent of oxidation has a much greater effect on the extent of degradation of a linear polysaccharide by an *exo*-enzyme than it has on a branched polysaccharide, in view of the greater number of chain ends in the latter.

The effect of various degrees of oxidation on the extent of degradation

- (65) S. A. Barker, G. I. Pardoe, M. Stacey, and J. W. Hopton, *Nature*, **197**, 231 (1963).
- (66) S. A. Barker, *Abstr. Intern. Symp. Carbohydr. Chem.*, 4th, Kingston, Ontario, 42 (1967).
- (67) S. A. Barker, G. I. Pardoe, M. Stacey, and J. W. Hopton, *Protides of the Biological Fluids*, Proc. Eleventh Colloq., Bruges, 1963, Elsevier, Amsterdam, 1964, p. 284.
- (68) S. A. Barker, G. I. Pardoe, M. Stacey, and J. W. Hopton, *Nature*, **204**, 938 (1964).
- (69) S. A. Barker, P. J. Somers, M. Stacey, and J. W. Hopton, *Carbohydr. Res.*, **1**, 106 (1965).
- (70) S. A. Barker, J. W. Hopton, P. J. Somers, and A. Repas, *Carbohydr. Res.*, **3**, 230 (1966).
- (71) J. J. Marshall and W. J. Whelan, unpublished work.

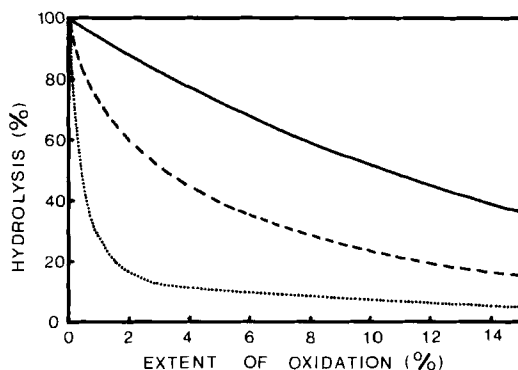


FIG. 3.—Action⁷¹ of *Rhizopus niveus* Glucoamylase (free from *alpha*-Amylase) on Samples of Glycogen (—), Amylopectin (---), and Amylose (.....), After Various Extents of Oxidation with Sodium Metaperiodate.

of linear amylose and the branched polysaccharides glycogen and amylopectin by crystalline glucoamylase free from *alpha*-amylase is shown in Fig. 3. It is readily apparent that the differences are sufficiently marked to permit distinction between the branched and linear polysaccharides.

d. Two-dimensional Chromatography Interspersed with Enzymic Reaction on the Paper.—French and coworkers⁷² have described a procedure suitable for qualitative examination of the nature of an oligosaccharide series produced by degradation of a polysaccharide. This involves separation of the oligosaccharides along one side of a large sheet of chromatography paper. After development, the area containing the sugars is sprayed with a solution of an enzyme, the action of which on the oligosaccharides is likely to yield some information regarding their structures. After incubation in a moist chamber for a convenient length of time, the chromatogram is developed at right angles to the first direction of development, together with suitable standards. Oligosaccharides not susceptible to the action of the enzyme are readily detected, as they are present on a diagonal after making the sugar zones visible. Enzyme action is apparent by the presence of mono- or oligo-saccharides moving, on the second development, ahead of the sugars on the diagonal. By comparison with the standards, and by taking account of the enzyme specificity, it is often possible to come to conclusions regarding the nature of the components of the starting mixture.

(72) D. French, A. O. Pulley, M. Abdullah, and J. C. Linden, *J. Chromatogr.*, **24**, 271 (1966).

Although this technique has been most widely used for examination of oligosaccharides of the type obtained from starch and glycogen, there is no reason why it should not be employed for examining the degradation products (enzymic or nonenzymic) of other types of polysaccharides. For illustrations of the results of the technique, see Refs. 73 and 74.

4. Problems Associated with the Use of Enzymes

The importance of enzyme purity has already been emphasized. When enzyme preparations are used that contain more than one enzyme acting on the substrate, or its degradation products, the results obtained will, at the best, be ambiguous and, more likely, completely meaningless. The same holds true when inadequately characterized enzymes are used.

A potential drawback associated with the use of some enzymes is the possibility of obtaining artifactual results through synthetic action of hydrolases, or contaminating activities in preparations of such enzymes. Such artifactual products may arise either from enzymic reversion, such as that occurring with almond emulsin (which gives a large number of β -D-linked oligosaccharides from D-glucose⁷⁵), or by transglycosylation wherein the glycon portion of the substrate is transferred to another sugar residue instead of to water, as in hydrolysis. Often, such reactions are more extensive when crude instead of purified enzymes are used, suggesting that they are caused by contaminating activities; the problem may be obviated by use of enzymes of higher purity. However, in other instances, the ability to catalyze such reactions appears to be an inherent property of the hydrolytic enzyme. Such appears to be true, for example, with testicular hyaluronidase, an enzyme well known for its transglycosylase activity.^{76,77}

Another point to be considered is the physical nature of the substrate. If this is an insoluble polysaccharide, the lack of attack by an enzyme is not very meaningful, as this may be due solely to the inability of the enzyme to act on the substrate in the insoluble form. Thus, paramylon, a (1 \rightarrow 3)- β -D-glucan from *Astasia ocellata*,⁷⁸ could not be degraded with a purified (1 \rightarrow 3)- β -D-glucanase from malt,^{78a} even though its structure is such that the enzyme should attack it. However, it is sometimes possible to circumvent this difficulty by conducting the enzymic reaction in

(73) K. Kainuma and D. French, *FEBS Lett.*, **5**, 257 (1968).

(74) M. Abdullah and D. French, *Arch. Biochem. Biophys.*, **137**, 483 (1970).

(75) S. Peat, W. J. Whelan, and K. A. Hinson, *Nature*, **170**, 1056 (1952).

(76) B. Weissmann, *J. Biol. Chem.*, **216**, 783 (1955).

(77) P. Hoffman, K. Meyer, and A. Linker, *J. Biol. Chem.*, **219**, 653 (1956).

(78) D. J. Manners, J. F. Ryley, and J. R. Stark, *Biochem. J.*, **101**, 323 (1966).

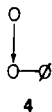
(78a) D. J. Manners and J. J. Marshall, *J. Inst. Brewing*, **75**, 550 (1969).

aqueous methyl sulfoxide,⁷⁹ in which many enzymes are active,⁸⁰ and in which the polysaccharide may be soluble even though it is insoluble in water.

The importance of rigorous characterization of any enzyme used for structural work is apparent from reports of the hydrolysis of linkages in polysaccharides, other than those expected to be susceptible, by certain glycoside hydrolases. Thus (1→4)- β -D-glucan hydrolases may also hydrolyze certain (1→4)- β -D-linked mannans and xylans. Although this property may be useful for examination of the structure of the "anomalous" substrate, it can sometimes lead to confusion, particularly where the polysaccharide under investigation contains two types of linkage possibly susceptible to enzyme action. Such is the case in the degradation of a glucomannan with (1→4)- β -D-glucanase.⁸¹

Lack of specificity of an enzyme for the linkage hydrolyzed in a "mixed-linkage" homopolysaccharide has already been mentioned (see p. 269), *Rhizopus arrhizus* laminaranase being able to split both (1→3)- and (1→4)- β -D-glucosidic linkages. The possibility that such an action may explain results difficult to account for on any other basis should always be borne in mind.

On some occasions, it is convenient to let two enzymes act on a substrate simultaneously; this may often be carried out successfully, for example, in the degradation of glycogen by simultaneous action of *beta*-amylase and pullulanase.⁴¹ The joint action of two enzymes may, however, produce unexpected results. Thus, an example has been reported where action of two β -D-glucan hydrolases on a polysaccharide (lichenan), which is a substrate for both, resulted in degradation by only one of the enzymes.⁸² In another example, a synergistic effect was noted during the simultaneous action of *alpha*-amylase and the transmalto-dextrinylase R-enzyme⁸³ on panose⁸⁴ (6²- α -D-glucosylmaltose, 4). Neither



(79) K. K. Tung and J. H. Nordin, *Anal. Biochem.*, **38**, 164 (1970).

(80) D. R. Lineback and A. L. Sayeed, *Carbohydr. Res.*, **17**, 453 (1971).

(81) K. Kato, T. Watanabe, and K. Matsuda, *Agr. Biol. Chem. (Tokyo)*, **34**, 532 (1970).

(82) A. S. Perlin, *Biochem. Biophys. Res. Commun.*, **16**, 538 (1965).

(83) S. Peat, W. J. Whelan, and W. R. Rees, *Nature*, **172**, 158 (1953); *J. Chem. Soc.*, 44 (1956).

(84) W. J. Whelan, *Biochem. Soc. Symp.*, **11**, 17 (1953).

of these enzymes, separately, has any action on this oligosaccharide. Simultaneous action of the debranching enzymes isoamylase and pullulanase on *beta*-limit dextrins did not result in complete debranching of these substrates; the degradation was, however, complete after sequential use of the enzymes.^{84a}

When quantitative measurements of the extent of degradation are being performed, it is essential that the stability of the enzyme under the conditions used be checked. Cessation of hydrolysis could be due not to absence of any further susceptible linkages but to the loss of enzymic activity. If an enzyme is still active after hydrolysis ceases, it may reasonably be assumed that the former situation is in effect. Loss of enzyme activity may be due to several causes, including the presence of inhibitors in the substrate (an example being the inhibition of *alpha*- and *beta*-amylases by mercuric ions present in samples of glycogen extracted with aqueous mercuric chloride), or, merely, instability of the enzyme under the conditions used. In the latter situation, it is often possible to confer increased stability on the enzyme by alteration of the physical conditions, or by addition of stabilizing agents (for example, serum albumin and reduced glutathione are useful with certain *beta*-amylase preparations⁸⁵).

Where chemical treatments of a polysaccharide are to be performed before and after the action of an enzyme, the possibility of interference by constituents of the enzyme preparation must be taken into consideration. One obvious pitfall is likely to arise when the enzyme is itself a glycoprotein.⁸⁶ It will be clear that periodate-oxidation measurements or methylation analyses after treatment of a polysaccharide with an enzyme of this type are likely to be meaningless unless suitable controls are included.

In some instances, the apparent specificity of an enzyme appears to change when the enzyme is used at different concentrations. Action on its poorest substrate may be negligible at low concentrations of the enzyme, but significant at higher concentrations. Thus, meaningful and reproducible results for extents of degradation and nature of end-products cannot be obtained unless the concentration of enzyme used in the reaction is known. This consideration may seem obvious, but it is often ignored, as emphasized, for example, by the controversies⁸⁷ over the specificities of the commonly used enzymes *alpha*-amylase, *beta*-amylase, and R-enzyme. As regards the last enzyme, previous work suggesting that

(84a) J. J. Marshall and W. J. Whelan, *Arch. Biochem. Biophys.*, **161**, 234 (1974).

(85) G. J. Walker and W. J. Whelan, *Biochem. J.*, **76**, 264 (1960).

(86) For a detailed discussion of carbohydrate-containing enzymes, see J. H. Pazur and N. N. Aronson, Jr., *Advan. Carbohydr. Chem. Biochem.*, **27**, 301 (1972).

(87) W. J. Whelan, J. M. Bailey, and P. J. P. Roberts, *J. Chem. Soc.*, 1293 (1953).

it contains separate activities acting on oligosaccharide and polysaccharide substrates^{88,89} was shown to be incorrect.⁹⁰ The conflicting results were probably accountable for on the basis of the use of different concentrations of the same enzyme. Action of sweet-potato *beta*-amylase on amylopectin and glycogen may result in two possible limit-dextrins, depending on whether the enzyme is used at high or low concentration.^{90a}

There is a most important factor to be considered when different enzymes are used to degrade a substrate sequentially. When enzymes are used in this way, it is generally important to inactivate the first enzyme before adding the second, as, otherwise, spurious results may be obtained due to the action of the second enzyme in exposing further residues susceptible to the action of the first enzyme. When carrying out this inactivation, which is generally achieved by heating, any heat stability of the enzyme being denatured should be investigated, and conditions chosen that are adequate to ensure destruction. Commonly used enzymes showing a high degree of heat stability include the debranching enzyme pullulanase⁹¹ and testicular hyaluronidase.⁹²

The final factor to be mentioned is the importance of taking precautions against microbial contamination of enzyme digests, particularly when incubations are of extended duration. Protection can be achieved by the presence of toluene or an antibiotic substance in the reaction mixture, but care should be taken to ensure that these agents do not interfere with analytical measurements.

III. ANALYTICAL METHODS UTILIZING ENZYMES

Enzymic procedures for the estimation of simple mono- and oligosaccharides released by chemical or enzymic treatment of polysaccharides, (for example, estimation of D-glucose by using D-glucose oxidase, or with hexokinase and D-glucose 6-phosphate dehydrogenase) have been recorded elsewhere.⁹³⁻⁹⁵ This Section will deal mainly with the ways in which these, and some related, enzymic procedures can be used in con-

(88) I. C. MacWilliam and G. Harris, *Arch. Biochem. Biophys.*, **34**, 442 (1959).

(89) D. J. Manners and K. L. Sparra, *J. Inst. Brewing*, **72**, 360 (1966).

(90) G. S. Drummond, E. E. Smith, and W. J. Whelan, *FEBS Lett.*, **9**, 136 (1970).

(90a) E. Y. C. Lee, *Arch. Biochem. Biophys.*, **146**, 488 (1971).

(91) M. Abdullah, B. J. Catley, W. F. J. Cuthbertson, and W. J. Whelan, *Biochem. J.*, **100**, 8P (1966).

(92) E. W. Emmart and J. B. Longley, *J. Gen. Physiol.*, **37**, 361 (1954).

(93) "Methods of Enzymatic Analysis," H. U. Bergmeyer, ed., Verlag Chemie, Weinheim, 1963.

(94) G. G. Guilbault, *Anal. Chem.*, **42**, 334R (1970).

(95) P. R. Finch, R. Yuen, H. Schachter, and M. A. Moscarello, *Anal. Biochem.*, **31**, 296 (1969).

junction with nonenzymic or other enzymic methods to obtain important, quantitative data relevant to the elucidation of polysaccharide structure. Other analytical procedures will be considered at more appropriate stages in this article.

1. Determination of Polysaccharides by Total Enzymic Hydrolysis to Monosaccharides

The classical method for determination of polysaccharides involved hydrolysis by acid to monosaccharides, followed by measurement of reducing power. Modifications of the procedure, especially with glucans, have used enzymic methods for determination of the monosaccharides liberated. In a few instances, it has proved possible to go one stage farther, and also to degrade the polysaccharides to the constituent monosaccharides enzymically (instead of by acid hydrolysis). The advantage of such a procedure is the speed and ease of performing the determination, particularly when large numbers of samples are involved. Additional advantages are the small amount of starting material necessary and the absence of partial decomposition of monosaccharides that may occur during acid hydrolysis.⁹⁶⁻⁹⁸ The principle of the method is best illustrated by a consideration of the determination of amylaceous polysaccharides by degradation to D-glucose with glucoamylase.^{99,100} The D-glucose liberated by enzyme action is then determined by using D-glucose oxidase.⁵⁸

It is rather important that the glucoamylase used be carefully tested, not in this instance for impurities, but because the presence of an *impurity* is essential if the procedure is to work satisfactorily! It has been shown¹⁰⁰ that use of crystalline *Rhizopus niveus* glucoamylase, which is free from *alpha*-amylase, results in considerably less than quantitative hydrolysis (as determined by acid hydrolysis). Table I shows the extents of conversion of a number of starch and glycogen samples into D-glucose by this highly purified enzyme, from which it is seen that conversions as low as 78% were obtained. Addition of *alpha*-amylase, or use of an *alpha*-amylase-contaminated preparation of glucoamylase from *Aspergillus niger*, resulted in quantitative conversions, suggesting the presence in the substrates of structural features that glucoamylase itself is unable to attack. For quantitative determination of amylaceous polysaccharides in

(96) S. J. Pirt and W. J. Whelan, *J. Sci. Food Agr.*, **2**, 224 (1951).

(97) F. H. Newth, *Advan. Carbohydr. Chem.*, **6**, 83 (1951).

(98) D. E. LaBerge and W. O. S. Meredith, *Lab. Pract.*, **19**, 1121 (1970).

(99) E. Y. C. Lee and W. J. Whelan, *Arch. Biochem. Biophys.*, **116**, 162 (1966).

(100) J. J. Marshall and W. J. Whelan, *FEBS Lett.*, **9**, 85 (1970).

TABLE I

D-Glucose Released Enzymically from Samples of Starch and Glycogen¹⁰⁰

Substrate	Glucose measured by enzymic hydrolysis ^a		
	<i>Aspergillus niger</i> glucoamylase	<i>Rhizopus niveus</i> glucoamylase	<i>Rhizopus niveus</i> glucoamylase + <i>alpha</i> -amylase
Potato amylose	97.0	90.1	101.0
Waxy-maize starch	100.0	97.6	103.0
Waxy-sorghum starch	98.0	92.6	100.0
Flordean starch	90.8	77.8	97.5
<i>beta</i> -Dextrin from waxy-maize starch	97.7	91.1	100.0
Shellfish glycogen	99.2	98.1	99.3
Sweet-corn phyto glycogen	99.9	96.2	100.3
Rabbit-liver glycogen	98.9	98.4	100.1
Skate-liver glycogen	95.3	89.3	99.3
Human-muscle glycogen	96.3	87.8	99.4
Rabbit-muscle glycogen	98.2	93.2	99.3
Cat-liver glycogen	93.1	82.2	98.8
<i>beta</i> -Dextrin from shellfish glycogen	94.5	92.7	97.0

^a Expressed as % of D-glucose released by acid; acid hydrolysis figures are corrected for D-glucose decomposed.

our laboratories, a not-too-highly purified sample of *Aspergillus niger* glucoamylase^{100a} to which *alpha*-amylase has been added to ensure complete degradation is routinely used.

This method, namely, quantitative enzymic conversion into monosaccharides, is applicable to any polysaccharide, provided that a suitable enzyme, or mixture of enzymes, can be found that will carry out the conversion. It is important to confirm, as with the glucoamylase hydrolysis, that quantitative degradation does occur under the conditions used. An attempt to develop a method for determination of soluble β -D-glucans and β -D-linked oligosaccharides by use of such enzyme preparations as almond emulsin was unsuccessful,¹⁰¹ the difficulty being attributable to the amount of reversion occurring.⁷⁵ It is, however, likely that the procedure could be modified by performing the degradation in the presence of D-glucose oxidase, so that the D-glucose liberated is immediately oxidized, and synthesis of oligosaccharides is precluded or reversed.

(100a) M. Y. Qureshi, Ph. D. Thesis, University of London (1967).

(101) J. J. Marshall, unpublished work.

2. Enzymic Determination of Products of Periodate Oxidation and Smith-Degradation Analysis

Smith degradation¹⁰² of oligo- and poly-saccharides is a most important and widely used chemical technique for the structural analysis of polysaccharides. The reactions involved in this method result in the production of alditols. Measurement of these products of the reaction, in particular, yields important structural information, and it is therefore essential that accurate analytical methods for alditols be available. Enzymic methods have been developed for the determination of two of the common alditols encountered namely, glycerol¹⁰³ and erythritol.¹⁰⁴ The advantage of these specific, enzymic techniques that use purified enzymes is the high degree of sensitivity, amounts of less than 0.1 μ mole of alditol being readily estimated. The determination may be performed directly on a sample of the reaction mixture after Smith degradation, without separation of the components and without the consequent decreased accuracy that separation incurs.

For both alditols mentioned, the method of estimation depends on the action of specific kinases, namely, erythritol kinase¹⁰⁴ and glycerol kinase,¹⁰³ that specifically phosphorylate the respective alditol. Both reactions are coupled with other enzymic reactions involving a change in the oxidation state of nicotinamide adenine dinucleotide, present as a cofactor. Thus, the extent of reaction may be measured, and the amount of alditol quantitated, by measurement of the u.v. absorbance at 340 nm. The sequences of reactions involved in the determinations are shown in Scheme 1(a,b). Although there do not appear to be any reports of the enzymic determination of glycolaldehyde after Smith degradation, this could conveniently be performed by using yeast alcohol dehydrogenase¹⁰⁵ (see Scheme 1c).

Formic acid is produced during periodate oxidation of glycans; its measurement may be used to determine the average chain length ($\overline{\text{c.l.}}$) of a branched polysaccharide, as one molecule is released from every nonreducing chain-end. Formic acid may be estimated accurately by using enzymic procedures; this was originally accomplished by conversion of formate into 10-formyltetrahydrofolic acid by the action of the enzyme formyltetrahydrofolate synthetase¹⁰⁶ (see Scheme 1d), the product

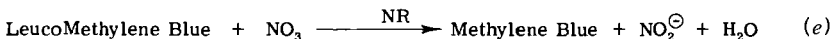
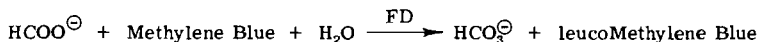
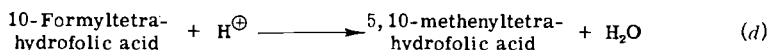
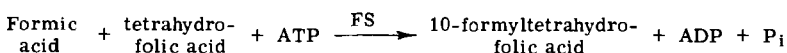
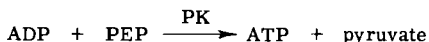
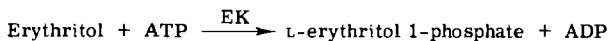
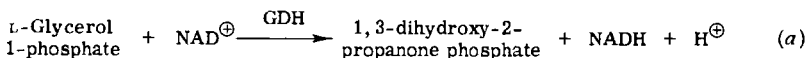
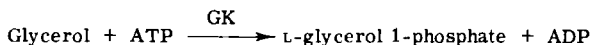
(102) I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, **5**, 361 (1965).

(103) D. W. Noble and R. J. Sturgeon, *Carbohydr. Res.*, **12**, 448 (1970).

(104) R. J. Sturgeon, *Carbohydr. Res.*, **17**, 115 (1971).

(105) H. Holzer, H. W. Goedde, and S. Schneider, *Biochem. Z.*, **327**, 245 (1955).

(106) D. H. Rammner and J. C. Rabinowitz, *Anal. Biochem.*, **4**, 116 (1962).



Scheme 1.—The Reactions Involved in the Enzymic Determination of (a) Glycerol, (b) Erythritol, (c) Glycolaldehyde, and (d) and (e) Formic Acid. [Abbreviations: ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-pyrophosphate; NAD⁺ and NADH, the oxidized and reduced forms of nicotinamide adenine dinucleotide; PEP, enolpyruvate phosphate; P_i, inorganic orthophosphate; GK, glycerol kinase; GDH, α-glycerophosphate dehydrogenase; EK, erythritol kinase; PK, pyruvate kinase; YAD, yeast alcohol dehydrogenase; FS, formyltetrahydrofolate synthetase; FD, formate dehydrogenase; and NR, nitrate reductase.]

being determined spectrophotometrically by measuring the increase in absorption at 350 nm resulting from the acid-catalyzed conversion of 10-formyltetrahydrofolic acid into 5,10-methenyltetrahydrofolic acid. An alternative procedure (see Scheme 1e) utilizes the enzymes formate dehydrogenase and nitrate reductase.¹⁰⁷ The product, nitrite, is determined spectrophotometrically.

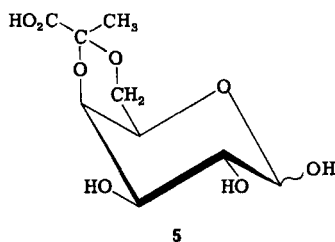
(107) E. Itagaki and S. Suzuki, *J. Biochem.* (Tokyo), **56**, 77 (1964).

3. Determination of the Degree of Polymerization of Glucans

A method for the determination of the number-average d.p. of glucans has been developed¹⁰⁸ that is based on the principle of the nonenzymic method first used by Peat and coworkers;¹⁰⁹ it involves reduction of the glucan with sodium borohydride, so that all reducing-end D-glucose residues are converted into D-glucitol. The enzymic adaptation involves hydrolysis of the reduced glucan with acid, followed by measurement of D-glucose and D-glucitol, the latter by means of the enzyme D-glucitol dehydrogenase. The oxidation of D-glucitol is accompanied by reduction of nicotinamide adenine dinucleotide, allowing a convenient spectrophotometric assay. This method could equally well be applied to the estimation of the d.p. of other glycans by using appropriate alditol dehydrogenases.

4. Assay of Pyruvic Acid in Agar and Other Polysaccharides

Agar has long been known to contain pyruvic acid,¹¹⁰ present in the form of the 4,6-O-(1-carboxyethylidene) derivative (5) of D-galactose.



It has also been shown to be present in the same, or a similar, form in certain microbial polysaccharides (for example, colanic acid¹¹¹).

One nonenzymic method¹¹⁰ for the determination of pyruvic acid is complicated and insensitive. Another method¹¹² is highly inaccurate unless a correction factor is applied.¹¹³ The most convenient and accurate method is based on the enzymic measurement of pyruvate after its release by mild hydrolysis with oxalic acid. The determination is conveniently performed with lactate dehydrogenase.¹¹³

- (108) D. J. Manners, A. J. Masson, and R. J. Sturgeon, *Carbohydr. Res.*, **17**, 109 (1971).
- (109) S. Peat, W. J. Whelan, and J. G. Roberts, *J. Chem. Soc.*, 2258 (1956).
- (110) S. Hirase, *Bull. Chem. Soc. Jap.*, **40**, 959 (1967).
- (111) C. J. Lawson, C. W. McCleary, H. I. Nakada, D. A. Rees, I. W. Sutherland, and J. F. Wilkinson, *Biochem. J.*, **115**, 949 (1969).
- (112) J. H. Sloneker and D. G. Orentas, *Nature*, **194**, 478 (1962).
- (113) M. Duckworth and W. Yaphe, *Chem. Ind. (London)*, 747 (1970).

IV. STARCH, GLYCOGEN, AND PULLULAN

The amylaceous polysaccharides constitute the best example of the successful application of enzymic techniques. The fundamental, structural features of these polysaccharides were established nonenzymically, to a large extent by Haworth and Hirst, before any enzymic degradation studies were undertaken. Subsequent use of well characterized enzymes, particularly by Peat and Whelan and their coworkers, has led to the elucidation of the fine structures.

The enzymic analysis of the structures of the amylaceous polysaccharides illustrates the applications of an enzyme which has been one of the most widely used in polysaccharide structural analysis, namely, *beta*-amylase. The availability of pure, crystalline preparations of this enzyme heralded the beginning of a new era in the structural analysis of polysaccharides which, as evidenced by new work in the field (for a review, see Ref. 12), is still gathering momentum.

A related polysaccharide, pullulan, is conveniently discussed together with the amylaceous polysaccharides, as the enzymes used in the elucidation of its structure are the same as those used for starch and glycogen.

1. Enzymes Involved in the Catabolism of Starch and Glycogen

a. *alpha*-Amylase.—This is one of the best-known starch-degrading enzymes, and highly purified preparations are readily obtained from a number of sources. For example, methods for the preparation of crystalline *alpha*-amylase from human saliva,¹¹⁴ the fungus *Aspergillus oryzae*,¹¹⁵ the bacterium *Bacillus subtilis*,¹¹⁶ and porcine pancreas¹¹⁵ are documented in the literature. The last two enzymes are available commercially in crystalline form. These crystalline enzymes are generally free from all other carbohydrases, although the occasional presence of maltase has been noted.¹¹⁷ In view of the ready availability of these crystalline enzymes, mention of others would be superfluous and their use unwise.

The properties and general specificity of *alpha*-amylase are well established.^{118,119} There are slight differences in the properties of the enzymes from different sources, but all hydrolytically depolymerize the starch-type polysaccharides in an essentially random manner (at least

(114) E. H. Fischer and E. A. Stein, *Biochem. Prepn.*, **8**, 27 (1961).

(115) E. H. Fischer and E. A. Stein, *Arch. Sci. (Geneva)*, **7**, 131 (1954).

(116) E. A. Stein and E. H. Fischer, *Biochem. Prepn.*, **8**, 34 (1961).

(117) H. Toda and S. Akabori, *J. Biochem. (Tokyo)*, **53**, 102 (1963).

(118) W. J. Whelan, *Staerke*, **12**, 358 (1960).

(119) J. F. Robyt and W. J. Whelan, in "Starch and Its Derivatives," J. A. Radley, ed., Chapman and Hall, London, 1968, p. 430.

in the initial stages of the reaction). The end products at high concentrations of the enzyme are D-glucose and maltose, together with a series of "alpha-limit dextrans." These dextrans are oligosaccharides of d.p. 4, or more, that contain the (1→6)-α-D-glucosidic linkages constituting the branch points in the substrate. As well as being themselves resistant to hydrolysis by alpha-amylases, the (1→6)-α-D-glucosidic linkages also stabilize, against hydrolysis by the enzyme, some of the (1→4)-α-D-glucosidic linkages in the region of the branch points. For this reason, isomaltose is not a product of the action of alpha-amylase. Early reports^{120,121} of the production of the latter oligosaccharide by the enzyme are erroneous, the isomaltose probably being produced by transferase impurities in the alpha-amylase preparations used. Some of the limit dextrans formed are multiply branched, that is, they contain more than one (1→6)-α-D-glucosidic linkage;^{122,123,123a} from glycogen, macromolecular dextrans may be obtained.¹²⁴⁻¹²⁷ The significance of these results will be discussed later (see p. 314). The consequence of the production of limit dextrans and of the differing proportions of (1→6)-α-D-glucosidic linkages in amylose, amylopectin, and glycogen is that there are considerable differences in the extents of hydrolysis of these substrates by alpha-amylase. Typical values are given in Table II.

All alpha-amylases require calcium ions for stability, or activity, or both.³⁸ The mammalian enzymes require chloride ions also.^{128,129} It is, therefore, important that these cofactors be incorporated into α-amylolysis digests. Where sequential use of alpha-amylase and other starch-degrading enzymes is being made, it is advisable to bear in mind the stable nature of alpha-amylase, especially in the presence of calcium ions, and to make sure that heating adequate to inactivate this enzyme is applied. The calcium-sequestering agent (ethylenedinitrilo)tetraacetic acid (EDTA) may also be of use for this purpose.

In contrast to some of the other starch-degrading enzymes, the random

- (120) E. M. Montgomery, F. B. Weakley, and G. E. Hilbert, *J. Amer. Chem. Soc.*, **71**, 1682 (1949).
- (121) K. H. Meyer and W. P. Gonon, *Helv. Chim. Acta*, **34**, 308 (1951).
- (122) P. J. P. Roberts and W. J. Whelan, *Biochem. J.*, **76**, 246 (1960).
- (123) D. French, *Bull. Soc. Chim. Biol.*, **42**, 1677 (1960).
- (123a) D. French, *Biochem. J.*, **100**, 2r (1966).
- (124) A. Levitzki and M. Schramm, *Bull. Res. Council Israel, Sect. A*, **11**, 258 (1963).
- (125) J. Heller and M. Schramm, *Biochim. Biophys. Acta*, **81**, 96 (1964).
- (126) A. Levitzki, J. Heller, and M. Schramm, *Biochim. Biophys. Acta*, **81**, 101 (1964).
- (127) A. Loyter and M. Schramm, *J. Biol. Chem.*, **241**, 2611 (1966).
- (128) K. Myrbäck, *Z. Physiol. Chem.*, **14**, 53 (1926).
- (129) G. J. Walker and W. J. Whelan, *Biochem. J.*, **76**, 257 (1960).

TABLE II
Typical Values for the Extents of Degradation of Amylaceous Polysaccharides
by Various Enzymes

Enzyme(s)	Degradation, measured as conversion into	Extent of degradation (%)		
		Amylose	Amylo- pectin	Glycogen
<i>alpha</i> -Amylase	maltose	110-120	85-95	75-85
<i>beta</i> -Amylase	maltose	70-80	50-60	40-50
Phosphorylase	α -D-glucosyl phosphate	~70	35-45	20-30
Glucoamylase containing traces of <i>alpha</i> -amylase	glucose	100	100	100
Pure glucoamylase	glucose			
from <i>Rhizopus niveus</i>		~90	80-100	80-100
from <i>Aspergillus niger</i>		n.d. ^a	65-75	40-50
Pullulanase	glucose	+ ^b	4-5	0 ^c
Isoamylase	glucose	+ ^b	4-5	7-10
<i>beta</i> -Amylase + pullulanase (used concurrently)	maltose	95-100	100	100
<i>beta</i> -Amylase + pullulanase (used successively)	maltose	85-100	95-100	40-50 ^c
<i>beta</i> -Amylase + isoamylase (used successively)	maltose	90-100	100-105	105-110

^a N.d. means "not determined."

^b Action can only be detected indirectly; for example, by increase in the *beta*-amylolysis limit. The symbol + indicates that hydrolysis occurs.

^c This is only true for certain samples of glycogen, presumably those that have not been degraded during isolation. For others, up to 50% of the branch points may be hydrolyzed by pullulanase, with a consequent increase in the *beta*-amylolysis limit of the glycogen.

and relatively unspecific action of this enzyme has rendered it of comparatively minor importance in structural analysis.

b. *beta*-Amylase.—This plant enzyme has been prepared in crystalline form from soybeans,¹³⁰ wheat,¹³¹ malted barley,¹³² and sweet potatoes.^{133,133a} The cereal enzymes tend to be contaminated with traces of an *alpha*-amylase-like impurity (Z-enzyme¹³⁴) and should therefore always be tested for this *endo*-acting carbohydrase; for example, by use of the

(130) J. Fukumoto and V. Tsujisaka, *Kagaku to Kogyo* (Osaka), **28**, 282 (1954).

(131) K. H. Meyer, P. F. Spahr, and E. H. Fischer, *Helv. Chim. Acta*, **36**, 1924 (1953).

(132) K. H. Meyer, E. H. Fischer, and A. Piguet, *Helv. Chim. Acta*, **34**, 316 (1951).

(133) A. K. Balls, M. K. Walden, and R. R. Thompson, *J. Biol. Chem.*, **173**, 9 (1948).

(133a) S. Nakayama and S. Amagase, *J. Biochem.* (Tokyo), **54**, 375 (1963).

(134) S. Peat, G. J. Thomas, and W. J. Whelan, *J. Chem. Soc.*, 722 (1952).

oxidized-amylose substrate described previously (see p. 266). Crystalline, sweet-potato *beta*-amylase as supplied by Worthington has been shown to contain a maltose-splitting impurity that interferes with analytical measurements.²⁹ This activity was originally suppressed by addition of a polyhydroxy compound, such as erythritol or Tris,^{32a} but a method has now been developed for complete removal of this contaminant.¹³⁵ A twice-recrystallized, maltase-free *beta*-amylase will soon be available commercially.

The activity and stability of sweet-potato *beta*-amylase are worthy of mention. In contrast to the original preparation of Balls and coworkers,¹³³ the commercial enzyme preparation was found to lose activity rapidly, particularly in dilute solution.⁸⁵ For this reason, it has become routine practice to add reduced glutathione and human-serum albumin¹³⁶ to stabilize the enzyme. However, the inaccuracies caused by the use of glutathione when quantitative measurements of the production of D-glucose are being performed have been pointed out.¹³⁷ Comparison of preparations of *beta*-amylase made in our laboratory¹³⁵ with those supplied by Worthington shows our preparation to have superior stability, as well as an improved (2–3 times as high) specific activity. Incubation of this enzyme in the absence of substrate or any stabilizing agent, at a concentration of 100 International units per ml (the usual concentration for *beta*-amylolysis digests) for 24 hrs at 37° resulted in little loss of activity, and on incubation under the same conditions at 2.5 units/ml, 30–40% of the activity was retained.^{137a} It is therefore no longer considered necessary to add stabilizing agents. As far as is known, *beta*-amylase shows no requirement for any metal ions or other cofactors for activity.

beta-Amylase acts on (1→4)- α -D-glucan chains by removing maltose units in a stepwise fashion from the nonreducing ends. Linear chains containing even numbers of D-glucose residues are, therefore, completely converted into β -maltose; linear chains having odd numbers of D-glucose residues yield, in addition, a single maltotriose residue from each reducing end.⁸⁴ The maltotriose is then slowly broken down to give maltose and D-glucose. Action of *beta*-amylase on the branched polysaccharides amylopectin and glycogen stops in the region of the (1→6)- α -D-glucosidic linkages. It is this feature of *beta*-amylase that makes it so useful, because it may be used to remove virtually all that part of these molecules to the exterior of their outermost branch-points, and leave the inner portions

(135) J. J. Marshall and W. J. Whelan, *Anal. Biochem.*, **52**, 642 (1973).

(136) Commercial, bovine-serum albumin preparations contain *alpha*-amylase or maltase activity, or both.

(137) D. M. Kilburn and P. M. Taylor, *Anal. Biochem.*, **27**, 555 (1969).

(137a) J. J. Marshall, unpublished work (1971).

of the molecules intact. The different proportions of (1→6)- α -D-glucosidic linkages in amylose, amylopectin, and glycogen result in marked differences in the extents of hydrolysis to maltose by the enzyme (Table II, p. 287).

The details of the behavior of the enzyme at branch points in polysaccharide chains have been examined in some detail. Side chains are trimmed, depending on whether they contain even or odd numbers of D-glucose residues, to leave (1→6)- α -D-linked maltosyl or maltotriosyl groups, respectively;^{138,139} (1→6)-linked α -D-glucosyl residues are not produced.

The question concerning the action of the enzyme on the main chain has caused some controversy.^{140,141} Current conclusions are that, when acting on the native polysaccharides, the action of the enzyme stops at one or two D-glucose residues from the branch point, so that the structures at the outermost branch-points in the product are as shown in Fig. 4. However, *beta*-amylase has considerably different action on model substrates containing (1→6)-linked α -D-glucosyl side-chains. Here, the action of the enzyme on the main chain either stops one D-glucose residue from the branch point, or hydrolyzes the linkage to the D-glucose residue involved in the branch point.¹⁴² The difference is the result of the smaller degree of steric hindrance caused by the α -D-glucosyl side-chain to the action of the enzyme.

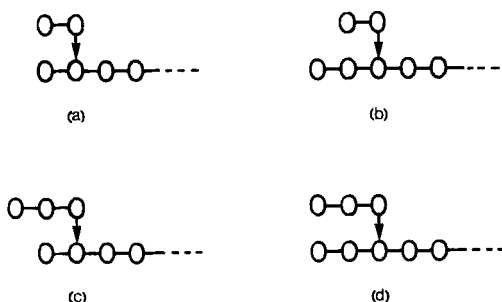


FIG. 4.—Structures Around the Outermost Branch-points in the Limit Dextrins Remaining After Action of *beta*-Amylase on Amylopectin and Glycogen. [Structure (b) is obtained uniquely by *beta*-amylase action on phosphorylase limit-dextrins (see p. 315). For an explanation of the symbols, see footnote 49.]

(138) S. Peat, W. J. Whelan, and G. J. Thomas, *J. Chem. Soc.*, 4546 (1952).

(139) R. Summer and D. French, *J. Biol. Chem.*, **222**, 469 (1956).

(140) D. French, *Baker's Dig.*, **31**, 24 (1957).

(141) D. French, in "Control of Glycogen Metabolism," W. J. Whelan and M. P. Cameron, eds., Churchill, London, 1964, p. 7.

(142) K. Kainuma and D. French, *FEBS Lett.*, **6**, 182 (1970).

Large-scale *beta*-amylolysis for the preparation of *beta*-amylase limit-dextrins¹⁴³ results in production of very large amounts of maltose, which tends to inhibit the enzyme.¹⁴⁴ To ensure complete *beta*-amylolysis, it is, therefore, usual to perform an initial degradation with enzyme to release the majority of the maltose from the exterior chains, and this is then removed by dialysis. The dialyzed product is then re-incubated with fresh enzyme to complete the degradation.¹⁴³ The small proportion of maltose produced during the second stage is not sufficient to cause any significant inhibition of the enzyme. Alternatively, the reaction may be performed in a dialysis bag, with continuous removal of the low-molecular-weight product. French⁴⁰ has commented on the difficulty of preparing true *beta*-amylase limit dextrins, that is, those that do not release more maltose on re-incubation with enzyme. In our laboratory, this has never been regarded as a significant problem, the extent of hydrolysis of *beta*-limit dextrins by *beta*-amylase being generally less than 1%. The problems experienced by French may be associated with low levels of enzyme used in the preparation of the *beta*-dextrins,^{90a} failure to relieve inhibition of the enzyme by dialysis of the maltose, or loss of enzymic activity before complete degradation,⁸⁵ or attributable to the presence of contaminating activities in the *beta*-amylase preparations used. It is also noteworthy that the rate of action of the enzyme on glycogen is considerably lower than on amylopectin (presumably for steric reasons), so that the *beta*-dextrins of the former polysaccharide are not so readily formed.^{90a}

Although *beta*-amylase has a reputation for being thermally labile, probably an extrapolation from the fact that a heat treatment is used to inactivate the enzyme during preparation of cereal *alpha*-amylases,¹⁴⁵ two observations suggest that this is not always true. Abdullah¹⁴⁶ has shown that, after preparation of a *beta*-limit dextrin and heating for the prescribed period of time (15 min) to inactivate the *beta*-amylase, enzymic activity still remained, presumably due to stabilization of the enzyme by the presence of polysaccharide. French has also reported¹⁴⁷ that, on two-dimensional chromatography interspersed with reaction on the paper (see p. 275) (in this instance, by spraying with *beta*-amylase), heating the chromatogram at 100° did not inactivate the enzyme. Failure to be aware of this possibility could well lead to spurious results.

The question of whether all chains in the branched molecules glycogen

(143) W. J. Whelan, *Methods Carbohydr. Chem.*, **4**, 261 (1964).

(144) U. K. Misra and D. French, *Biochem. J.*, **77**, 1F (1960).

(145) E. Kneen, R. M. Sandstedt, and C. M. Hollenbeck, *Cereal Chem.*, **20**, 399 (1943).

(146) M. Abdullah, unpublished work.

(147) D. French, personal communication (1971).

and amylopectin are, in fact, accessible to *beta*-amylase¹⁴⁰ has not as yet been answered. This important point is considered later in this article (see p. 318).

c. Glucoamylase.—This is a microbial enzyme, produced by molds of the *Aspergillus*,^{148,149} *Rhizopus*,¹⁵⁰ *Endomyces*,¹⁵¹ and other species, that acts on amylaceous polysaccharides and derived oligosaccharides, releasing molecules of β -D-glucose in a stepwise fashion from the non-reducing chain-ends. Crude culture-filtrates are contaminated with interfering enzymes,^{148,152,153} particularly glucosidases, transferases, and *alpha*-amylase, that must be removed before use of the enzyme.¹⁵⁴ For the *Aspergillus niger* enzyme, it has been reported that these contaminants may be eliminated by column chromatography on O-(2-diethylaminoethyl)cellulose in a variety of ways.^{148,152,153,155} Glucoamylase, a glyco-enzyme,⁵⁶ is eluted as two fractions differing in carbohydrate content.^{155a,156} The *Rhizopus* enzyme is also a glycoprotein, but it does not exist in multiple forms.¹⁵⁰ The discussion of this enzyme will be restricted mainly to the most-studied *Aspergillus* enzyme, although some comparisons with the *Rhizopus* enzyme will be made where appropriate.

It has been reported several times that purification of the *Aspergillus* isoenzymes by pH gradient chromatography on O-(2-diethylaminoethyl)-cellulose results in pure glucoamylase (see, for example, Ref. 153). By using enzyme preparations made by this and similar procedures to examine the substrate specificity, it has been concluded that the enzyme is one of the least specific of the starch-degrading enzymes. Thus, it has been reported repeatedly^{148,155,157,158} that the *Aspergillus* enzyme brings about complete hydrolysis of the substrates to D-glucose, indicating its

- (148) J. H. Pazur and T. Ando, *J. Biol. Chem.*, **234**, 1966 (1959).
- (149) D. R. Lineback and W. E. Baumann, *Carbohydr. Res.*, **14**, 341 (1970).
- (150) J. H. Pazur and S. Okada, *Carbohydr. Res.*, **4**, 371 (1967).
- (151) T. Fukui and Z. Nikuni, *Agr. Biol. Chem.* (Tokyo), **33**, 884 (1969).
- (152) I. D. Fleming and B. A. Stone, *Biochem. J.*, **97**, 13P (1965).
- (153) D. R. Lineback, I. J. Russell, and C. Rasmussen, *Arch. Biochem. Biophys.*, **134**, 539 (1969).
- (154) With the possible exception of *alpha*-amylase, where the preparations are to be used for the estimation of amylaceous polymers (see p. 280).
- (155) K. L. Smiley, D. E. Hensley, M. J. Smiley, and H. J. Gasdorf, *Arch. Biochem. Biophys.*, **144**, 694 (1971).
- (155a) J. H. Pazur, K. Kleppe, and E. M. Ball, *Arch. Biochem. Biophys.*, **103**, 515 (1963).
- (156) J. H. Pazur, H. R. Knull, and A. Cepure, *Carbohydr. Res.*, **20**, 83 (1971).
- (157) J. H. Pazur and T. Ando, *J. Biol. Chem.*, **235**, 297 (1960).
- (158) J. H. Pazur, in "Starch, Chemistry and Technology," R. L. Whistler and E. F. Paschall, eds., Academic Press, New York, 1965, Vol. 1, p. 133.

ability to split both (1→4)- and (1→6)- α -D-glucosidic linkages in these polysaccharides. In addition, it has been stated to act on (1→6)- and (1→3)- α -D-glucosidic linkages in oligosaccharides.¹⁵⁹

Work in this laboratory revealed the presence of at least one impurity, *alpha*-amylase, in such preparations.^{34,35,87,100} Although this contaminant is necessary when the enzyme is to be used for determination of amylaceous polymers,¹⁰⁰ it renders specificity and structural studies based on such preparations meaningless. Further purification¹⁶⁰ resulted in the removal of this impurity, and, possibly, others also, from one of the iso-enzyme fractions. The purified enzyme then produced a limit dextrin from amylopectin in the proportion of ~30% of the original polysaccharide. Amylopectin *beta*-limit dextrin was hydrolyzed to the extent of only 30% by the enzyme. These values are consistent with the theory that action of the enzyme is impeded by the outermost branch points in these polysaccharides, although the purified enzyme still appears to be able to hydrolyze the (1→6)- α -D-glucosidic linkages in such *linear* oligosaccharides as panose (4; see p. 277). Such highly purified preparations of glucoamylase are likely to be of great importance in the structural analysis of amylaceous polysaccharides and for the examination of derived oligosaccharides. As yet, however, the nature of the limit dextrin produced from amylopectin has not been characterized, except insofar as to show that the A-chains¹⁶¹ remaining are single, D-glucose stubs.¹⁶⁰

It may be that the specificity of the *Rhizopus niveus* enzyme, which is available commercially in a crystalline, *alpha*-amylase-free form,¹⁶² is different, as similar concentrations of it resulted in considerably higher extents of hydrolysis of amylaceous polysaccharides.¹⁰⁰ The lack of complete hydrolysis by the latter enzyme could be accounted for by the presence of "anomalous" groupings or arrangements in the substrate, rather than by (1→6)- α -D-glucosidic linkages hindering enzyme action. However, although *alpha*-amylase has been shown to be absent from this preparation, the possible presence of a separate enzyme splitting (1→6)- α -D-glucosidic linkages must be considered.

The main drawback associated with the use of glucoamylase is the formation of reversion products,^{163,164} particularly isomaltose; this necessitates taking some care in interpretation of results. Although this reaction

(159) J. H. Pazur and K. Kleppe, *J. Biol. Chem.*, **237**, 1002 (1962).

(160) J. J. Marshall and W. J. Whelan, *Abstr. 9th Int. Congr. Biochem., Stockholm*, 103 (1973).

(161) A-Chains are side chains; B-chains are main chains (see p. 310).

(162) Research Products Division, Miles Laboratories, Inc., Elkhart, Indiana.

(163) T. Watanabe, S. Kawamura, H. Sasaki, and K. Matsuda, *Staerke*, **21**, 18 (1969).

(164) T. Watanabe, S. Kawamura, H. Sasaki, and K. Matsuda, *Staerke*, **21**, 44 (1969).

has been presumed to be an inherent property of the enzyme, this presumption remains to be confirmed. This problem may not be experienced when highly purified preparations of the enzyme are used. Glucoamylase is a highly stable enzyme,¹⁶⁵ and so it is easy to use; and it does not appear to require any cofactors.

d. Debranching Enzymes.—Debranching enzymes cleave the (1→6)- α -D-glucosidic inter-chain linkages in amylopectin, glycogen, and certain derived oligosaccharides.¹⁶⁶ Such enzymes have been of great importance in analysis of the structures of these polysaccharides. The debranching enzymes will be discussed under three headings: (i) R-enzyme and pullulanase, (ii) isoamylase, and (iii) glucosidase-transferase. The last enzyme system differs from the enzymes in the other two categories in that two enzymes are necessary for debranching and they act by an indirect mechanism, instead of by direct debranching. A summary of the specificities of these enzymes is presented in Table III.

(i) **Pullulanase and R-Enzyme.**¹⁶⁷—These enzymes are discussed together, because the present evidence is such as to suggest that their specificities are closely similar, possibly even to the extent of being identical.^{90,166}

R-Enzyme was first isolated from broad beans and potatoes by Peat and coworkers.¹⁶⁸ Purification was difficult in view of the relatively small amounts present and the large number of contaminating enzymes, particularly a maltodextrinyl transferase (D-enzyme),⁸³ branching (Q-) enzyme,¹⁶⁹ and *alpha*-amylase. However, a procedure was developed¹⁶⁸ that gave preparations sufficiently pure to permit routine use of this enzyme during some ten years for debranching of amylaceous polysaccharides.¹²

At the concentration used, debranching of amylopectin and its *beta*-limit dextrin was incomplete,¹⁶⁸ and there was no action on glycogen.¹⁷⁰ First-stage, *alpha*-amylase limit-dextrins, those containing side chains of

(165) J. H. Pazur, H. R. Knull, and D. L. Simpson, *Biochem. Biophys. Res. Commun.*, **40**, 110 (1970).

(166) E. Y. C. Lee and W. J. Whelan, *Enzymes*, **5**, 191 (1971).

(166a) J. J. Marshall, *Wallerstein Lab. Commun.*, **35**, 49 (1972).

(166b) D. J. Manners, *Nature New Biol.*, **234**, 150 (1971).



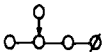
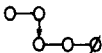
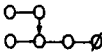
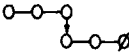
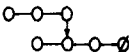
(167) In this article, R-enzyme is referred to in its original sense. A claim⁹⁰ that it consists of two separate activities, one active towards polysaccharides and the other, towards oligosaccharides, has been shown to be incorrect.⁹⁰

(168) P. N. Hobson, W. J. Whelan, and S. Peat, *J. Chem. Soc.*, 1451 (1951).

(169) P. N. Hobson, W. J. Whelan, and S. Peat, *J. Chem. Soc.*, 3566 (1950).

(170) S. Peat, W. J. Whelan, P. N. Hobson, and G. J. Thomas, *J. Chem. Soc.*, 4440 (1954).

TABLE III
Specificities^{166a} of Debranching Enzymes^a

Substrate	R-Enzyme			Iso- amylase	Gluco- sidase- Transferase
	Sweet corn	Potato	Pullulanase		
<i>Polysaccharides</i>					
Amylopectin	+	+	+	+	+
Glycogen	—	—	— ^b	+	+
Amylopectin ϕ -dextrin	+	n.d.	+	+	+
Glycogen ϕ -dextrin	+	n.d.	+	+	+
Amylopectin <i>beta</i> -dextrin	+	+	+	+	+
Glycogen <i>beta</i> -dextrin	+	—	+	+	+
Pullulan	+	+	+	—	—
<i>Oligosaccharides</i>					
First-stage, <i>alpha</i> -limit dextrin mixture ¹²⁰	+	+	+	+	+
	+	n.d.	+	—	—
	n.d.	+	n.d.	n.d.	n.d.
	—	—	—	—	+
	+	+	+	—	—
	+	+	+	—	—
	+	+	+	—	n.d.
	+	+	+	+	+

^a The symbol + indicates that a polysaccharide or oligosaccharide is hydrolyzed by the debranching enzyme; — indicates no hydrolysis. No attempt has been made to show the limiting extents of debranching of the various substrates by the debranching enzymes. Thus, + should not be taken to indicate that complete debranching takes place, but rather that action (partial or complete) on the substrate is observable. Attempts to designate a substrate as being readily hydrolyzed or slowly hydrolyzed^{166b} are meaningless without a statement of the conditions used.⁹⁰ N.d. indicates "not determined." ^b Only true for undegraded glycogens (compare Table II, p. 287).

two or more D-glucose residues,¹²⁹ were substrates for the enzyme. Much later work⁹⁰ showed the hydrolysis of pullulan by the enzyme, confirming a proposed similarity between this enzyme and bacterial pullulanase.⁴¹

Pullulanase was discovered by Bender and Wallenfels,¹⁷¹ after isolation (171) H. Bender and K. Wallenfels, *Biochem. Z.*, 334, 79 (1961).

and culture of an air-borne bacterium, identified as *Aerobacter aerogenes*, capable of growing on solutions of pullulan. Several methods for purification of the enzyme have been described, the most successful involving growth of the organism under conditions where the enzyme remains cell-bound, followed by extraction from the cells with detergent.^{24,172,173} Pullulanase has been crystallized,^{24,172} and the enzyme prepared in this way is now used in preference to R-enzyme for structural studies.

The specificity of this enzyme is such that it acts on amylopectin to bring about almost complete (generally, ~95%) debranching.^{24,41} It completely debranches amylopectin *beta*-limit dextrin, releasing large proportions of maltose, maltotriose, and higher oligosaccharides. Glycogen *beta*-limit dextrin is partly debranched, glycogen not at all (or to a very limited extent), and first-stage, *alpha*-amylase limit-dextrins, virtually completely.^{24,41} Action of the enzyme on pullulan proceeds in an *endo*, not an *exo*, fashion, so that (1→6)-linked polymers of α -maltotriose, mainly of d.p. 6, 9, 12 . . . , may be isolated in addition to maltotriose.⁹⁰ The mode of action on branched polysaccharides appears, however, to be predominantly *exo*. Action of the enzyme on glycogen seems to take place only with certain samples of this polysaccharide, perhaps reflecting a limited degradation during their isolation. Thus, high-molecular-weight rabbit-liver glycogen²⁴ and phytoglycogen,¹⁷⁴ both isolated in the presence of mercuric chloride (to minimize enzymic degradation), are not significantly hydrolyzed by the enzyme. In contrast, degradation of these two polysaccharides by pullulanase and *beta*-amylase acting together, when the effective substrate for the debranching enzyme is glycogen *beta*-limit dextrin, results in complete hydrolysis to maltose. The optimum length of side chain hydrolyzed by the enzyme is 2 or 3 D-glucose residues, so that amylopectin *beta*-limit dextrin and *alpha*-limit dextrins are the best substrates for the enzyme.²⁴ A similar situation exists with plant R-enzyme;⁹⁰ this is in clear contrast to the situation with isoamylase (see the next subsection). The slight differences apparent in specificity between pullulanase and R-enzyme, for example, in their behavior towards glycogen, may result merely from the differences in concentration at which their specificities have been ascertained (compare Ref. 90).

One important point to be remembered when pullulanase is being used is its heat stability.⁹¹ Treatment of digests at 100° for a short time is not sufficient to render the enzyme inactive; activity returns spontaneously on standing. This is more noticeable at pH 7 than at pH 5. It is most impor-

(172) K. Wallenfels, H. Bender, and I. R. Rached, *Biochem. Biophys. Res. Commun.*, **22**, 254 (1966).

(173) H. Bender and K. Wallenfels, *Methods Enzymol.*, **8**, 555 (1966).

(174) J. J. Marshall, to be published.

tant that complete inactivation of pullulanase be achieved when subsequent *beta*-amylolysis is to be performed, because any residual pullulanase activity may cause anomalously high extents of conversion into maltose.

(ii) **Isoamylase.**—Although the existence in yeast of an enzyme capable of debranching glycogen has been known for many years,¹⁷⁵ this enzyme has never been purified, and its specificity is still unknown. In addition, discovery of a second debranching enzyme in yeast^{176,177} (of the glucosidase-transferase type) has rendered much of the early work with yeast isoamylase suspect. For these reasons, yeast isoamylase will not be discussed as extensively as the more recently discovered and better characterized isoamylases.

Harada and coworkers¹⁷⁸ in Japan, and Whelan and coworkers⁴² in the U.S.A., independently discovered isoamylases in other organisms. Culture filtrates of a species of *Cytophaga* examined in this laboratory have been found to contain an isoamylase free from any other starch-degrading enzyme activity. The Japanese group obtained their enzyme from an unidentified species of *Pseudomonas* that is not freely available. Both of these isoamylases are readily freed from contaminating protein (and, for the *Cytophaga* enzyme, carbohydrate also) present in the crude culture-filtrates, giving preparations suitable for use in structural work. Thus, a two-step purification procedure involving column chromatography on *O*-(2-diethylaminoethyl)cellulose and Biogel P-60 resulted in over 100-fold purification of the *Cytophaga* enzyme.^{43,48} Chromatography on Sephadex G-100 provides an alternative method for purifying the enzyme readily.¹⁷⁹ As yet, the enzyme has not been crystallized. In contrast to pullulanase, the enzyme is not markedly heat-stable, and it may readily be inactivated after use. No cofactors or ion requirements have been noted, but its inhibition⁴⁸ by EDTA may suggest the involvement of a cation for activity, or stabilization, as observed with pullulanase.^{180,181}

The specificity of isoamylase is such that it completely debranches

(175) B. Maruo and T. Kobayashi, *Nature*, **167**, 606 (1951).

(176) E. Y. C. Lee, L. D. Nielsen, and E. H. Fischer, *Arch. Biochem. Biophys.*, **121**, 245 (1967).

(177) E. Y. C. Lee, J. H. Carter, L. D. Nielsen, and E. H. Fischer, *Biochemistry*, **9**, 2347 (1970).

(178) K. Yokobayashi, A. Misaki, and T. Harada, *Biochim. Biophys. Acta*, **212**, 458 (1970).

(179) J. J. Marshall, unpublished work (1973).

(180) E. Y. C. Lee, Ph. D. Thesis, University of London, 1966.

(181) B. J. Catley and C. J. Moyes, unpublished work.

glycogens, amylopectins, and the phosphorylase limit-dextrins of these polysaccharides.^{42,178} Rather surprisingly (at first), the action of the enzyme on *beta*-limit dextrins of glycogen and amylopectin was found to be incomplete.^{42,178} This behavior was soon traced to the inability of the enzyme to hydrolyze side chains of 2 D-glucose residues (which constitute half of the side chains in these dextrins¹³⁸), and this has proved to be one of the most useful properties of the enzyme. This specificity also results in the lack of substantial degradation of first-stage, *alpha*-limit dextrins. A further specificity requirement of this enzyme is that the (1→6)- α -D-glucosidic linkage hydrolyzed must be at a true branch-point, that is, to the primary hydroxyl group of a D-glucose residue linked through both O-1 and O-4. Thus, the enzyme is without action on the linear polysaccharide pullulan. This is one of the most definitive ways of distinguishing between enzymes of the isoamylase and pullulanase-R-enzyme types. Figure 5 shows a detailed comparison of the minimum specificity requirements of these two types of debranching enzyme.

(iii) **Glucosidase-Transferase.**—This enzyme [first known as amylo-(1→6)-glucosidase¹⁸² and now accorded the systematic name amylo-(1→6)- α -D-glucosidase oligo-(1→4)→(1→4)- α -D-glucan transferase, E.C. 3.2.1.33] has a long history of use in the analysis of amylopectin and glycogen structures. The hydrolytic component of the enzyme removes single, (1→6)-linked, α -D-glucose residues attached to (1→4)- α -D-glucan chains. Only long after the discovery of the enzyme was it realized that this is a “double-headed” enzyme or a binary enzyme-complex, the other

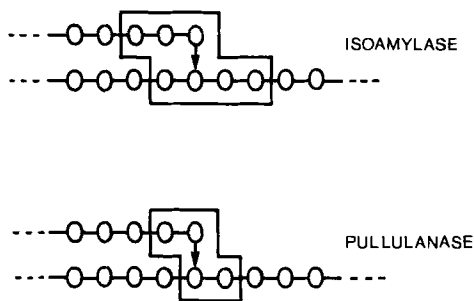


FIG. 5.—Diagrammatic Representation of the Structural Requirements for Action of *Cytophaga* Isoamylase; and Comparison with Those of Pullulanase. [The smallest substrates currently known for these two enzymes are 6³- α -maltotriosylmaltotetraose and 6⁵- α -maltosylmaltose, respectively.]

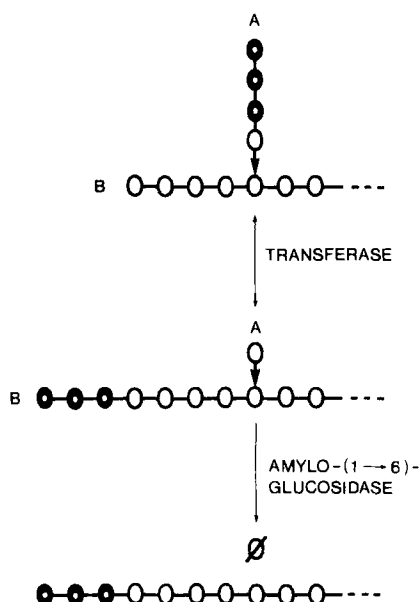


FIG. 6.—Diagrammatic Representation of the Debranching of Phosphorylase Limit-dextrin by Glucosidase-Transferase.

component being a (1→4)- α -D-glucan transferase which, in most instances, is involved in the debranching process.^{183,184} Side chains of 3 or more D-glucose residues (optimal length 4) are shortened to a single D-glucose residue by action of the transferase, which can move a segment from the side chain to the main chain (see Fig. 6). The exposed D-glucose residue is then hydrolyzed by the amylo-(1→6)-glucosidase component of the enzyme system.

The enzyme was first extracted from rabbit muscle,¹⁸² but was subsequently isolated from other sources, including yeast.^{176,177} The mammalian and yeast enzyme systems have similar properties and mechanisms of action.¹⁷⁷ There is no apparent requirement for cofactors, but certain buffers (for example, Tris) are unsuitable for use with this enzyme.^{185,186} One noteworthy phenomenon is the reputed ability to inactivate one of the two components selectively.¹⁸⁵ In the presence of molybdate, amylo-

(183) M. Abdullah and W. J. Whelan, *Nature*, 197, 979 (1963).

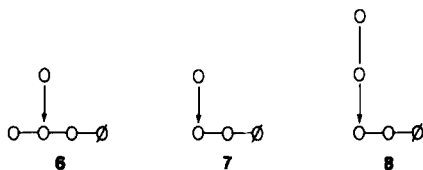
(184) D. H. Brown, B. Illingworth, and C. F. Cori, *Nature*, 197, 980 (1963).

(185) T. E. Nelson, D. H. Palmer, and J. Larner, *Biochim. Biophys. Acta*, 212, 269 (1970).

(186) D. H. Brown and B. I. Brown, *Methods Enzymol.*, 8, 515 (1966).

(1→6)-glucosidase activity may be utilized without any complications due to the action of transferase. This property could be of important application in structural studies.

It is probable that, *in vivo*, the enzyme system acts together with phosphorylase in the utilization of glycogen,^{85,187} and, in structural studies, it has been of most value when used in conjunction with the latter enzyme. Both the mammalian and yeast debranching-enzyme systems are most active on phosphorylase limit-dextrins. With these substrates, the debranching occurs as depicted in Fig. 6, a maltotriose residue being transferred before the single D-glucose residue is released. The specificity of the transferase is such that action on the *beta*-limit dextrins of glycogen and amylopectin is slow and incomplete,¹⁶⁶ because an unfavored maltosyl transfer is required. With the native, undegraded polysaccharides, the action is again slow and incomplete, multiple transfers being necessary to expose a (1→6)-linked α -D-glucose residue. Action on *alpha*-limit dextrins necessitates that the A-chain¹⁶¹ be a single D-glucose residue at a branch point, the smallest substrate being 6³- α -D-glucosylmaltotetraose¹⁸⁸ (6). There is no action on 6³- α -D-glucosylmaltotriose (7) or 6³- α -maltosylmaltotriose (8). Some of the mistaken ideas regarding the



action of the enzyme will become apparent during the discussion of its application in structural studies.

e. Phosphorylase.—This enzyme has been prepared from several sources—plant, microbial, and mammalian.^{189–192} It is only necessary to discuss the rabbit-muscle enzyme, as this is one of the most readily

- (187) M. Abdullah, P. M. Taylor, and W. J. Whelan, in "Control of Glycogen Metabolism," W. J. Whelan and M. P. Cameron, eds., Churchill, London, 1964, p. 123.
- (188) B. Illingworth and D. H. Brown, *Proc. Nat. Acad. Sci. U. S.*, **48**, 1619 (1962).
- (189) M. Cohn, *Enzymes*, **5**, 179 (1961).
- (190) D. H. Brown and C. F. Cori, *Enzymes*, **5**, 207 (1961).
- (191) J. Holló, E. László, and Á. Hoschke, "Plant α -1,4-Glucan Phosphorylase," Akadémiai Kiadó, Publishing House of the Hungarian Academy of Sciences, Budapest, 1971.
- (192) E. H. Fischer, A. Pocker, and J. C. Saari, in "Essays in Biochemistry," P. N. Campbell and F. Dickens, eds., Academic Press, London, 1970, Vol. 6, p. 23.

purified and most frequently used enzymes employed in analysis of the fine structures of amylopectin and glycogen.

The enzyme acts on amylaceous polysaccharides by liberating non-reducing-end D-glucosyl residues in an *exo* fashion as α -D-glucosyl phosphate. The reaction is reversible, and, for this reason, it is necessarily carried out in a high concentration of inorganic orthophosphate, to ensure complete degradation. Alternatively, when phosphorylase limit (ϕ)-dextrins are being prepared, a dialysis method in phosphate buffer can be used;^{192a} this is similar to the method for preparation of *beta*-amylase limit-dextrins discussed earlier (see p. 290).

The enzyme from rabbit muscle may be extracted and crystallized readily,¹⁹³ but many recrystallizations may be necessary to ensure freedom from such contaminants as *alpha*-amylase, debranching enzyme, and branching enzyme. Purification may be facilitated by the use of column chromatography on *O*-(2-diethylaminoethyl)cellulose.¹⁹⁴ The enzyme exists in two forms (*a* and *b*) that differ in their requirement for adenosine 5'-monophosphate as cofactor. The *b* form is inactive in the absence of cofactor, and may be converted into the active (adenosine 5'-monophosphate-independent) *a* form by phosphorylation of a specific serine residue under the action of (contaminant-free) phosphorylase kinase with adenosine 5'-triphosphate as the phosphate donor.

The enzyme has been most useful in the examination of branched polysaccharides, from which it gives a limit dextrin (ϕ -dextrin). The action of the enzyme, like that of *beta*-amylase and *Aspergillus niger* glucoamylase, is halted in the region of the branch points. The specificity of the enzyme is such that, on reaching the limit of hydrolysis, 4 D-glucose residues are left in both the A-chains and outer B-chains.⁸⁵ However, this conclusion was only reached by a devious route, and was proved by elegant, enzymic investigations of the structure of the limit dextrin which will be discussed later (see p. 312).

One property of phosphorylase that has not been widely used, but that could often be advantageous, is its ability to degrade α -D-glucans, releasing D-glucose instead of D-glucosyl phosphate when the reaction is conducted in the presence of arsenate rather than phosphate.¹⁹⁵ It is considered that D-glucosyl arsenate is formed initially, but that it is labile

(192a) H. G. Hers, W. Verhue, and F. van Hoof, *Eur. J. Biochem.*, **2**, 257 (1967).

(193) E. H. Fischer and E. G. Krebs, *J. Biol. Chem.*, **231**, 65 (1958).

(194) F. Huijing, E. Y. C. Lee, J. H. Carter, and W. J. Whelan, *FEBS Lett.*, **7**, 251 (1970).

(195) J. Katz and W. Z. Hassid, *Arch. Biochem.*, **30**, 272 (1951).

and quickly hydrolyzed. The advantages of using this procedure are the ease of determining the liberated D-glucose and the fact that hydrolysis of the arsenic ester means that the equilibrium otherwise produced is disturbed, and so degradation proceeds to completion. It would seem that this latter effect could also be achieved by addition of phosphatase to phosphorolysis digests.

f. α -D-Glucosidases.—In contrast to glucoamylase, these enzymes are oligosaccharidases; the favored substrates are small malto-oligosaccharides, and the terminal, nonreducing-end, D-glucosyl groups are liberated as α -D-glucose. Few enzymes of this type have been reported, but those from yeasts^{32b,196} and sweet corn¹⁹⁷ have been obtained in high purity. The yeast enzymes hydrolyze maltose and maltotriose (*Saccharomyces cerevisiae*) or maltose only (*S. uvarum*). Neither acts on isomaltose, nor on higher malto-oligosaccharides. The sweet-corn enzyme differs in being able to degrade other oligosaccharides, such as panose, isomaltose, and kojibiose, but is also essentially inactive towards polysaccharide substrates.¹⁹⁸ The most important application of the yeast enzymes is for the specific determination of maltose or maltotriose, or both, in the presence of higher oligosaccharides and polysaccharides; this cannot be done with the *exo*-polysaccharidase glucoamylase, which degrades both oligosaccharide and polysaccharide substrates.

g. *Pseudomonas stutzeri* Amylase.—This is an enzyme¹⁹⁹ that has, as yet, had little application to the analysis of starch and glycogen structures. However, it is mentioned here, as it is of considerable potential importance in this field.

The enzyme is readily prepared, in an apparently pure form, from the culture filtrates of this pseudomonad, and has a specific activity comparable to that of crystalline, sweet-potato *beta*-amylase. The *exo* action of the enzyme is analogous to that of *beta*-amylase, but maltotetraose residues, not maltose residues, are removed. The action of the enzyme is halted by branch points, so that limit dextrans are produced from glycogen and amylopectin. The specificity of the enzyme for linkages in the region of branch points has not yet been ascertained. Some applications of this enzyme will be discussed later (see p. 318).

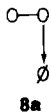
(196) A. W. Phillips, *Arch. Biochem. Biophys.*, **80**, 346 (1959).

(197) J. J. Marshall and P. M. Taylor, *Biochem. Biophys. Res. Commun.*, **42**, 173 (1971).

(198) J. J. Marshall and A. P. Iodice, to be published.

(199) J. F. Robyt and R. J. Ackerman, *Arch. Biochem. Biophys.*, **145**, 105 (1971).

h. Isopullulanase.—A preliminary report^{199a} described the isolation of this enzyme, obtained from the cells of a strain of *Aspergillus niger*, which degrades pullulan to isopanose (6¹- α -maltosylglucose; 8a) and which also



acts on panose (4; see p. 277) giving isomaltose and D-glucose. These results suggest that the enzyme specifically hydrolyzes (1→4)- α -D-glucosidic linkages from O-1 of D-glucose residues carrying substituents at O-6. Should this type of specificity be confirmed, the enzyme may prove of great utility for structural studies on amylopectin and glycogen, as it may allow fragmentation of these macromolecules and their degradation products in a manner not hitherto possible.

2. Use of Enzymes in the Structural Analysis of Starch and Glycogen

a. General.—Early chemical studies on starch, for the most part performed on the unfractionated material, established the fundamental features of its structure (for reviews, see Refs. 200–202). Thus, partial hydrolysis with acid, and methylation analysis, indicated that the molecules consist of (1→4)- α -D-glucan chains, and end-group estimations indicated the presence of 25–30 D-glucose residues per chain. To account for the measured molecular weight,^{203,204} which was considerably higher than that ($\sim 5,000$ daltons) expected for chains containing 25–30 D-glucose residues, it was suggested that the individual chains might be joined by linkages other than the (1→4)- α -D type,²⁰⁵ most probably (1→6)- α -D in nature.²⁰⁶ Shortly after these basic features had been established, it was

- (199a) Y. Sakano, N. Masuda, and T. Kobayashi, *Agr. Biol. Chem.* (Tokyo), 35, 971 (1971).
- (200) W. J. Whelan, in "Encyclopaedia of Plant Physiology," W. Ruhland, ed., Springer-Verlag, Berlin, 1958, Vol. 6, p. 154.
- (201) M. L. Wolfrom and H. (S.) El Khadem, in "Starch, Chemistry and Technology," R. L. Whistler and E. F. Paschall, eds., Academic Press, New York, 1965, Vol. 1, p. 251.
- (202) J. M. Williams, in "Starch and Its Derivatives," J. A. Radley, ed., Chapman and Hall, London, 1968, p. 91.
- (203) S. R. Carter and B. Record, *J. Chem. Soc.*, 660, 664 (1939).
- (204) E. L. Hirst and G. T. Young, *J. Chem. Soc.*, 1471 (1939).
- (205) W. N. Haworth, *Monatsh.*, 69, 314 (1936).
- (206) K. Freudenberg and H. Boppel, *Ber.*, 71, 2505 (1938).

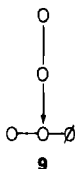
found that most starches could be separated into two fractions,²⁰⁷⁻²¹¹ the major component, amylopectin, being a branched polysaccharide whose features approximated to those originally ascribed to whole starch, but with a chain length of the order of 20-25 D-glucose residues.²¹²⁻²¹⁴ The second, minor component (amylose) was shown to be a linear polysaccharide consisting of (1→4)-linked α -D-glucosyl residues. The same type of chemical procedures showed that glycogen is closely similar to the branched component of starch, the only apparent difference being in the chain length, which is of the order of 10-14 D-glucose residues for the former polysaccharide.^{215,216} Action of *beta*-amylase on both starch and glycogen confirmed the (1→4)- α -D-linked nature of the chains, an oligosaccharide characterized as maltose being liberated from both.²¹⁷

Evidence that the D-glucosidic interchain linkages in starch are, in fact, (1→6)- α -D in nature came originally from the isolation of small proportions of 2,3-di-O-methyl-D-glucose after methylation and hydrolysis.^{218,219} This evidence was, however, hardly definitive, as the small amounts of this dimethyl sugar obtained could equally well have arisen as a result of undermethylation or demethylation. The isolation of isomaltose after the action of crude, bacterial *alpha*-amylase on the polysaccharides^{120,121} is equally equivocal. No *pure alpha*-amylase is known that gives isomaltose as a product of its action on starch,¹¹⁸ and it is likely that this product was formed by a contaminating enzyme in the crude preparation of *alpha*-amylase used.

Proof of the nature of the interchain linkages came from examination of the structures of the limit dextrins produced by the action of highly purified *alpha*-amylases on starch and glycogen.^{123a} These oligosaccharides contain both (1→4)- and (1→6)- α -D-glucosidic linkages, but no others. It has proved much easier to characterize the nature of the branch points

- (207) K. H. Meyer, W. Brentano, and P. Bernfeld, *Helv. Chim. Acta*, **23**, 845 (1940).
- (208) K. H. Meyer, P. Bernfeld, and E. Wolff, *Helv. Chim. Acta*, **23**, 854 (1940).
- (209) T. J. Schoch, *Cereal Chem.*, **18**, 121 (1941).
- (210) T. J. Schoch, *J. Amer. Chem. Soc.*, **64**, 2957 (1942).
- (211) T. J. Schoch, *Advan. Carbohydr. Chem.*, **1**, 247 (1945).
- (212) K. H. Meyer, M. Wertheim, and P. Bernfeld, *Helv. Chim. Acta*, **23**, 865 (1940).
- (213) K. Hess and B. Krajne, *Ber.*, **73**, 976 (1940).
- (214) W. Z. Hassid and R. M. McCready, *J. Amer. Chem. Soc.*, **65**, 1157 (1943).
- (215) K. H. Meyer, *Advan. Enzymol.*, **3**, 109 (1942).
- (216) D. J. Manners, *Advan. Carbohydr. Chem.*, **12**, 261 (1957).
- (217) K. Myrbäck, *Advan. Carbohydr. Chem.*, **3**, 251 (1948).
- (218) K. Freudenberg and H. Boppel, *Naturwissenschaften*, **28**, 264 (1940).
- (219) C. C. Barker, E. L. Hirst, and G. T. Young, *Nature*, **147**, 296 (1941).

by examination of these oligosaccharides instead of the native polysaccharides, as the *alpha*-amylase-resistant interchain-linkages are localized in these oligosaccharides. It should, however, be noted that, despite their comparatively small size, these oligosaccharides cannot always be characterized unequivocally by chemical analysis alone, supplementation of the chemical examination by enzymic methods sometimes being necessary, as illustrated for the pentasaccharide 6²- α -maltosylmaltotriose (9), pro-



duced by the action of *Bacillus subtilis* *alpha*-amylase on waxy-maize starch.^{220,221} This is a most important oligosaccharide, as it is one of the two smallest oligosaccharides known that exhibit all the structural features of the interchain linkage, the other being the isomeric pentasaccharide 6³- α -D-glucosylmaltotetraose (6; see p. 299), which is produced by other *alpha*-amylases, for example, salivary²²² and malted-cereal²²³ *alpha*-amylases. Periodate over-oxidation of the *B. subtilis* pentasaccharide and its alcohol yielded, respectively, 1 and 2 moles of formaldehyde per mole. On hydrolysis, the methylated polysaccharide gave 2,3-di-, 2,3,6-tri-, and 2,3,4,6-tetra-O-methyl-D-glucose in the ratios of 100:195:158. These results by themselves are, however, not sufficient to characterize the oligosaccharide, as they are consistent with either of the structures shown in Fig. 7. Enzymic evidence distinguished between them. The inability of *beta*-amylase to act on the pentasaccharide, together with the action of R-enzyme, which afforded maltose and maltotriose, excluded the structure shown in Fig. 7b, and showed that the pentasaccharide is 6²- α -maltosylmaltotriose (see Fig. 7a). Treatment of the limit dextrin with glucosylamylase, which has limited action on (1 \rightarrow 6)- α -D-glucosidic linkages, yielded only glucose and panose in the molar ratio of 2:0.85. Although this result is consistent with either of the structures depicted in Fig. 7, the two possibilities could be distinguished by examination of the intermediates in the hydrolysis. Two tetrasaccharides were separated by

(220) R. C. Hughes, E. E. Smith, and W. J. Whelan, *Biochem. J.*, **88**, 63P (1963).

(221) D. French, E. E. Smith, and W. J. Whelan, *Carbohydr. Res.*, **22**, 123 (1972).

(222) B. J. Catley, Ph. D. Thesis, University of London, 1967.

(223) D. J. Manners and J. J. Marshall, *Carbohydr. Res.*, **18**, 203 (1971).

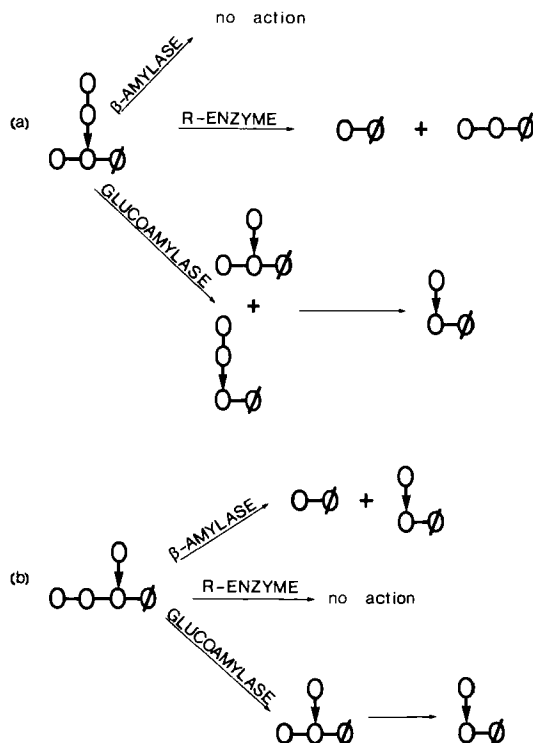


FIG. 7.—The Two Structures Possible for the *Bacillus subtilis* Pentasaccharide α -Limit Dextrin on the Basis of Chemical Evidence; and the Action of Various Enzymes on These Structures.

paper chromatography, confirming that the pentasaccharide is 6²- α -maltosylmaltotriose (see Fig. 7a); the alternative, 6²- α -D-glucosylmaltotetraose, would yield a single, intermediate tetrasaccharide (see Fig. 7b).

Although, on the basis of chemical studies, it was possible to ascertain the gross structures of the molecules of starch and glycogen, the use of highly purified enzymes has become established as one of the most versatile and definitive methods for the determination of the fine structure of these polysaccharides. The use of these techniques in the examination of the fine structures of glycogen, amylopectin, and amylose will now be discussed.

b. Fine Structure of Amylose.—Action of *beta*-amylase on amylose was shown to result in complete conversion into maltose.²¹⁴ Thus, it was

initially considered that this polysaccharide consisted entirely of chains of (1→4)-linked α -D-glucose residues lacking any fine structure. However, the availability of preparations of highly purified, crystalline, sweet-potato *beta*-amylase¹³³ led to reconsideration of this idea. Action of this purified enzyme on amylose resulted in conversions into maltose that were considerably less than quantitative, generally²²⁴⁻²²⁶ of the order of 70%. The undegraded portion of the polysaccharide stained blue with iodine,²²⁴⁻²²⁶ this being characteristic of linear (1→4)- α -D-glucan chains. The presence in the amylose molecules of blockages to the action of the *exo*-enzyme was, therefore, postulated, but the nature of these blockages, and of the contaminant (termed Z-enzyme) present in the amorphous *beta*-amylase preparations²²⁷ that enabled the anomalous linkages to be bypassed, has been the subject of a considerable amount of controversy.^{134,228,229} Only the former problem has as yet been satisfactorily solved.

The proposal that amylose contains non-(1→4)- α -D-glucosidic linkages was not readily accepted by some workers. Thus, Meyer and coworkers²³⁰ claimed that the results of Peat and coworkers²²⁴ were artifactual and were caused by a retrogradation of the amylose, before complete degradation, that rendered it immune to further hydrolysis. This hypothesis was disproved by the demonstration that aged solutions of amylose, with and without alkali treatment, are degraded by crystalline *beta*-amylase to identical extents.²²⁶ Neither was the incomplete hydrolysis due to inactivation of the enzyme. A second objection to be considered was the possibility that the results were attributable to the presence of amylopectin in the amylose samples.²³¹ However, calculations show that, for a degree of *beta*-amylolysis of 75%, there would have to be 35-40% of amylopectin present, and this would have been readily detected. In addition, the *beta*-limit dextrin of amylose affords a blue stain with iodine;²²⁴⁻²²⁶ that of amylopectin does not. A third possibility is that these blockages, apparently indicative of a fine structure, are merely modifications introduced during isolation of the polysaccharide or during subsequent treatments. Thus, it has been shown that heating of amylose

(224) S. Peat, W. J. Whelan, and S. J. Pirt, *Nature*, **164**, 499 (1949).

(225) G. J. Thomas, W. J. Whelan, and S. Peat, *Biochem. J.*, **47**, xl (1950).

(226) S. Peat, S. J. Pirt, and W. J. Whelan, *J. Chem. Soc.*, 705 (1952).

(227) S. Peat, S. J. Pirt, and W. J. Whelan, *J. Chem. Soc.*, 714 (1952).

(228) R. H. Hopkins and R. Bird, *Nature*, **172**, 492 (1953).

(229) S. Peat and W. J. Whelan, *Nature*, **172**, 494 (1953).

(230) K. H. Meyer, P. Bernfeld, R. A. Boissonnas, P. Gürtler, and G. Noelting, *J. Phys. Chem.*, **53**, 319 (1949).

(231) K. H. Meyer, *Experientia*, **8**, 405 (1952).

solutions at neutral pH (or, particularly, when alkaline) results in a significant diminution in the extent of *beta*-amylolysis.²³² Also, the *beta*-amylolysis of amylose samples prepared in the presence of nitrogen are considerably higher than those prepared in an atmosphere of oxygen.²³³ Undoubtedly, this is an important factor to be considered, and care must therefore be taken during preparation of amylose to avoid the introduction of blockages to *exo*-enzyme action (for example, by isolation under nitrogen, to avoid oxidative modification).

However, evidence in favor of a true structural feature that resists the action of *beta*-amylase has come from studies of the action of debranching enzymes on amylose and its *beta*-limit dextrin. Thus, by treatment with yeast isoamylase, the *beta*-amylolysis limit of a sample of amylose was increased from 76 to 90%, and that of amylose *beta*-limit dextrin²³⁴ from 6 to 77%. Treatment of amylose with pullulanase also increases the conversion of the substrate into maltose by *beta*-amylase to an almost quantitative value.²³⁵ On the basis of these results, the "anomalous" linkages in amylose that resist *beta*-amylase action are considered to be a very small proportion of (1 \rightarrow 6)- α -D-glucosidic linkages.

The nature of Z-enzyme, the contaminant in crude, soybean, *beta*-amylase preparations that is responsible for allowing quantitative degradation of amylose by *beta*-amylase, has not yet been ascertained with certainty. Peat and coworkers¹³⁴ were well aware that a small proportion of *alpha*-amylase would have exactly the same effect as Z-enzyme. The properties of the enzyme were, however, inconsistent with its being an *alpha*-amylase.²²⁷ Furthermore, the extents of hydrolysis of amylose by *beta*-amylase preparations containing Z-enzyme were greater than those achieved by using Z-enzyme-free *beta*-amylase to which *alpha*-amylase had been added, indicating that Z-enzyme cleaves the "anomalous" linkages, whereas *alpha*-amylase merely allows *beta*-amylase to bypass them.²²⁵ Both Manners²³⁶ and Greenwood²³⁷ and their coworkers have gone to the extent of pointing out the obvious, namely, that *alpha*-amylase *simulates* Z-enzyme in allowing an increased degree of *beta*-amylolysis, and have suggested that these enzymes are identical. Green-

(232) R. T. Bottle, G. A. Gilbert, C. T. Greenwood, and K. N. Saad, *Chem. Ind.* (London), 541 (1953).

(233) W. Banks, C. T. Greenwood, and J. Thomson, *Chem. Ind.* (London), 928 (1959).

(234) O. Kjølborg and D. J. Manners, *Biochem. J.*, **86**, 258 (1963).

(235) W. Banks and C. T. Greenwood, *Arch. Biochem. Biophys.*, **117**, 674 (1966).

(236) W. L. Cunningham, D. J. Manners, A. Wright, and I. D. Fleming, *J. Chem. Soc.*, 2602 (1960).

(237) W. Banks, C. T. Greenwood, and I. G. Jones, *J. Chem. Soc.*, 150 (1960).

wood and coworkers²³⁸ subsequently gave the name Z-enzyme to an enzyme, isolated from soybeans, that is clearly an *alpha*-amylase. This enzyme, however, had properties very different from those of the Z-enzyme examined by Peat and coworkers,²²⁷ particularly in heat-stability.²³⁸ Peat's Z-enzyme was certainly not a phosphatase. Although phosphoric ester groupings are present in starch^{210,227,238a} and are known to obstruct the action of *beta*-amylase,^{238a} addition of bone phosphatase did not cause any change in the *beta*-amylolysis limit of amylose by crystalline, sweet-potato *beta*-amylase.²²⁷ In view of the demonstrated effect of de-branching enzymes on amylose^{234,235} and the absence of any definitive evidence showing that Z-enzyme does not split (1→6)- α -D-glucosidic linkages, the nature of Z-enzyme remains unsettled.

Although the blockages to *beta*-amylase action have been demonstrated to be (1→6)- α -D-glucosidic linkages, these may be of two types, either linkages in a linear chain or inter-chain branch-points (see Fig. 8). It has not yet been ascertained which of these possibilities is correct, although the problem is of rather interesting biosynthetic significance. Should it prove that (1→6)- α -D-glucosidic linkages are present in a linear chain,

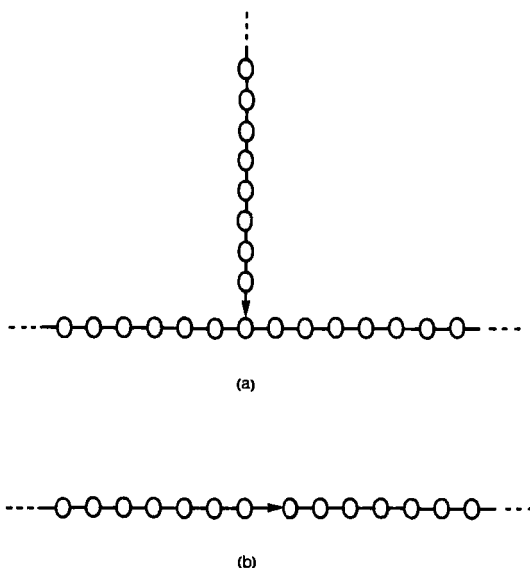


FIG. 8.—The Two Possible Arrangements of the (1→6)- α -D-Glucosidic Linkages in Amylose. [In (a), this linkage constitutes a branch point, and in (b), it is present as a linkage in a linear chain of D-glucose residues.]

(238) C. T. Greenwood, A. W. MacGregor, and E. A. Milne, *Carbohydr. Res.*, **1**, 229 (1965).

(238a) T. Posternak, *J. Biol. Chem.*, **188**, 317 (1951).

this feature would suggest that the chain-extending enzyme (amylose synthetase) is not entirely specific for transferring D-glucose to the secondary hydroxyl group at C-4 of the D-glucose residue at the growing chain-end. On the other hand, if these linkages are present as branch points, this would indicate that, during biosynthesis, the amylose component is accessible to the action of branching enzyme (Q-enzyme). It should be relatively easy to distinguish between these two possibilities by comparison of the effect of a well characterized isoamylase, such as that from *Cytophaga*,⁴² with the effect of pullulanase, as measured by *beta*-amylolysis after treatment with the debranching enzymes. (1→6)- α -D-Glucosidic linkages in a linear chain would not be hydrolyzed by isoamylase⁴³ and should, therefore, not result in any increase in *beta*-amylolysis of the amylose.

One further feature of the structure of amylose that has been disclosed by the use of enzymes is its heterogeneity. Extraction of amylose by aqueous leaching of starch at 70°, the method originally used for the fractionation of starch, gives only linear amylose, as evidenced by its complete degradation to maltose with *beta*-amylase.^{239,240} Extraction at a higher temperature (90–100°) then gives amylose containing the “anomalous” linkages, and this has a lower *beta*-amylolysis limit (50–60%). There is no reason to suspect that the fractionation results from factors other than differences in molecular weight, the less soluble fraction, containing the (1→6)- α -D-linkages, being larger.

c. Fine Structures of Amylopectin and Glycogen.—Three structures for these branched polysaccharides were considered possible on the supposition that the molecules consist of large numbers of relatively short chains joined by (1→6)- α -D-glucosidic linkages. These structures differed only in the way in which the unit chains were arranged. The three structures, proposed by Haworth and coworkers,²⁴¹ Staudinger and Husemann,²⁴² and Meyer and coworkers,^{243,244} are illustrated diagrammatically²⁴⁵ in Fig. 9(a–c). It was only possible to distinguish between these arrangements by the use of enzymic methods.

Peat and coworkers¹³⁸ distinguished the different types of chains in

(239) W. Banks and C. T. Greenwood, *Staerke*, **11**, 294 (1959).

(240) J. M. G. Cowie, I. D. Fleming, C. T. Greenwood, and D. J. Manners, *J. Chem. Soc.*, 4430 (1957).

(241) W. N. Haworth, E. L. Hirst, and F. A. Isherwood, *J. Chem. Soc.*, 577 (1937).

(242) H. Staudinger and E. Husemann, *Ann.*, **527**, 195 (1937).

(243) K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta*, **23**, 875 (1940).

(244) K. H. Meyer and M. Fuld, *Helv. Chim. Acta*, **24**, 375 (1941).

(245) These three structures are, generally, referred to simply as the Haworth, Staudinger, and Meyer structures, respectively, and this nomenclature will be used in this article.

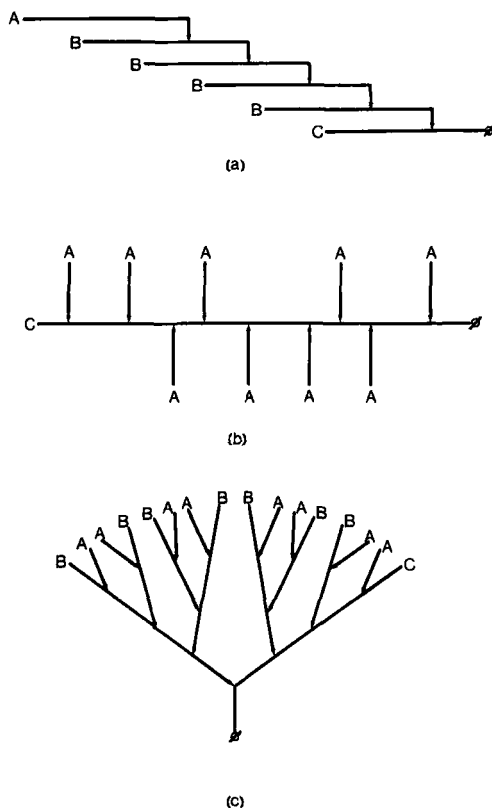


FIG. 9.—Diagrammatic Representations of the Structures Proposed for Amylopectin and Glycogen by Haworth and Coworkers (a), Staudinger and Husemann (b), and Meyer and Coworkers (c).²⁴⁸ [Straight lines represent chains of (1→4)-linked α -D-glucose residues, and arrowheads, (1→6)- α -D-glucosidic, interchain linkages. The nomenclature of the various chains is that of Peat and coworkers.¹²⁶]

the molecules as A, B, or C, A-chains being those bearing no substituent chains, B-chains being those carrying substituent chains linked to a proportion of their primary hydroxyl groups, and the C-chain being the single chain in the molecule having (presumably) a free reducing group. Consideration of the three structures proposed shows that the essential difference between them lies in the number of substituent chains that each chain in the molecule carries, that is, a difference in the proportions of A- and B-chains. In the Haworth structure, in which all chains carry one substituent chain, practically all of the chains are B-chains. In the Staudinger structure, on the other hand, all chains with the exception of the C-chain are unsubstituted and, hence, are A-chains. The Meyer

structure is more random, with the average degree of substitution of each chain lying somewhere between these extremes, A- and B-chains being present in similar numbers. Peat and coworkers¹³⁸ realized that the three structures could be distinguished on this basis, by direct measurement of the number of chains of each type. The difficulty lay, however, in knowing how to distinguish between the various types of chain. For amylopectin (waxy-maize starch), the problem was solved by treatment with *beta*-amylase and isolation of the *beta*-amylase limit-dextrin. In this dextrin, all of the A-chains are trimmed to 2 or 3 D-glucose residues, and, statistically, these two types of A-chain must be present in equal numbers. The B-chains, although themselves shortened by *beta*-amylase, are longer than the A-chains, the majority containing 6 or more D-glucose residues. Thus, the A-chains could be readily recognized after debranching of the *beta*-amylase limit-dextrin with R-enzyme. Fractionation of the debranched, *beta*-amylase limit-dextrin of waxy-maize starch was performed on a carbon-Celite column,^{87,246} and the maltose and maltotriose were separated from the higher oligosaccharides.¹³⁸ These two oligosaccharides accounted for 12.8% of the weight of the original *beta*-limit dextrin.²⁴⁷ As this value is intermediate between the proportions that would have been expected from a Haworth type of molecule (negligible) and a Staudinger type of molecule (20.8%) having the properties (c.l. 24; *beta*-amylolysis limit 52%) of the original amylopectin, it was concluded that the Meyer model best fits the structure of amylopectin. In view of the incomplete debranching²⁴⁸ of the *beta*-dextrin by R-enzyme, Peat and coworkers¹³⁸ did not attempt to draw any conclusions regarding the relative number of A- and B-chains present, although attempts to determine this were made by other workers²⁴⁹ using the values of Peat and coworkers.¹³⁸ Thus, it became apparent that an essential feature of the structure of amylopectin is the presence of multiple branching, that is, chains carrying more than one substituent chain.

In view of the resistance of glycogen and its *beta*-limit dextrin to hydrolysis by R-enzyme,¹⁷⁰ the fine structure of glycogen could not be investigated in a similar way. However, an attempt to show that this polysaccharide also fits the Meyer model was made by Cori and coworkers²⁵⁰ by using the enzymes phosphorylase and "amylo-(1 \rightarrow 6)-

(246) R. L. Whistler and D. F. Durso, *J. Amer. Chem. Soc.*, **72**, 677 (1950).

(247) S. Peat, W. J. Whelan, and G. J. Thomas, *J. Chem. Soc.*, 3025 (1956).

(248) The specificity of the enzyme is such that short chains are preferentially hydrolyzed,⁹⁰ so that it is probable that all of the maltose and maltotriose was released.

(249) E. L. Hirst and D. J. Mannors, *Chem. Ind. (London)*, 224 (1954).

(250) J. Larner, B. Illingworth, G. T. Cori, and C. F. Cori, *J. Biol. Chem.*, **199**, 641 (1952).

glucosidase" (glucosidase-transferase). Before considering their work, it is necessary to digress to consider the action of these enzymes on glycogen, and the structure of the phosphorylase limit-dextrin. Elucidation of this structure has, in turn, depended on the use of other enzymes.

Initial observations that muscle phosphorylase could degrade glycogen almost completely²⁵¹ were subsequently accounted for by the discovery of the debranching enzyme "amylo-(1→6)-glucosidase" in the preparation of phosphorylase used.¹⁸² Phosphorylase preparations free from this debranching enzyme gave a limit (ϕ -) dextrin amounting to some 60–80% of the original glycogen.²⁵² Amylo-(1→6)-glucosidase acts on this dextrin by releasing D-glucose residues and allowing degradation by phosphorylase to proceed further.¹⁸² Not unreasonably, it was concluded that the enzyme was removing (1→6)-linked α -D-glucose stubs present in the ϕ -dextrin. As the action of *beta*-amylase on the ϕ -dextrin resulted in removal of the equivalent of one maltose residue per chain end,¹⁸² it had to be assumed that, whereas the A-chain was a single D-glucose residue, the outer B-chain was 6 or 7 D-glucose residues long. Thus, the ϕ -dextrin was presumed to have the structure shown in Fig. 10a. Direct hydrolytic removal of the D-glucose stub was presumed to be involved in the removal of the barrier to phosphorylase action.

That this interpretation of the action of phosphorylase and the debranching enzyme is incorrect came later from the work of Walker and Whelan.⁸⁵ They examined the structure of the limit dextrin prepared by the action of highly purified, muscle phosphorylase on amylopectin. Debranching of this dextrin by R-enzyme resulted in production of maltotetraose. (Similar results were later obtained when glycogen ϕ -dextrin was debranched with pullulanase.¹⁸⁷) It was therefore concluded that the unequal attenuation of the two chains by phosphorylase, proposed

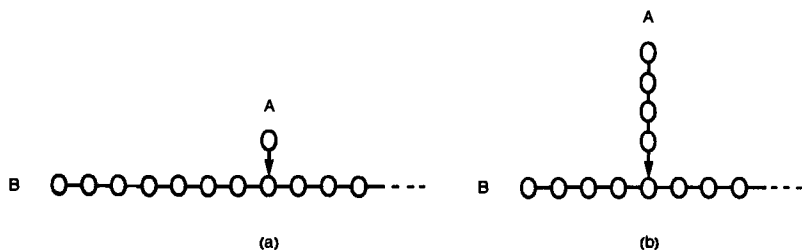


FIG. 10.—Arrangement of the D-Glucose Residues Around the Outermost Branch-points of Phosphorylase (ϕ)-Limit Dextrin, according to (a) Cori and Lerner,¹⁸² and (b) Walker and Whelan.⁸⁵ [The symbols are as given in footnote 49.]

(251) M. A. Swanson, *J. Biol. Chem.*, **172**, 805 (1948).

(252) S. Hestrin, *J. Biol. Chem.*, **179**, 943 (1949).

by Cori and Larner,¹⁸² does not take place. Instead, both the A-chains and the outer B-chains contain 4 D-glucose residues (see Fig. 10b), and *beta*-amylase removes a single maltose residue from each, instead of 2 from the B-chain.²⁶³

This discovery immediately raised questions regarding the mechanism of action of the amylo-(1→6)-glucosidase. The situation was rationalized by the proposal⁸⁵ that this enzyme acts by a two-stage mechanism involving (a) transfer of maltotriose from side (A) chain to main (B) chain to expose a single D-glucose residue, followed by (b) liberation of this residue by hydrolysis (see Fig. 6; p. 298). The hypothesis was later proved correct.^{183,184,253a} As a result of this work, it is clear that the evidence educed by Larner and coworkers²⁵⁰ to establish the Meyer structure for glycogen is invalid.

These workers²⁵⁰ submitted glycogen to enzymic degradation with phosphorylase to obtain the ϕ -dextrin. Action of amylo-(1→6)-glucosidase on this dextrin liberated D-glucose, and subsequent phosphorolysis yielded a second limit-dextrin. Repetition of this procedure was found to liberate, at each stage, a decreasing amount of D-glucose, and, after three such treatments, almost 90% of the original polysaccharide had been degraded. The two other structures possible would have yielded equal amounts of D-glucose at each debranching step. Thus, on the basis of this evidence for the apparent presence of tiers of branch points, obtained by assuming the wrong structure for the limit dextrin and an incorrect mechanism of action of the debranching enzyme, Larner and coworkers²⁵⁰ considered that the Meyer structure had been proved for glycogen. The arguments against the definitive nature of their results have been discussed by Lee and Whelan,¹⁰⁶ and are as follows. It was assumed that all of the A-chains, and only the A-chains, were removed on treatment with the debranching-enzyme system. This assumption is not valid, because the reversible nature of the transferase component results in extension, not removal, of some A-chains. In addition, some B-chains are converted into A-chains after removal of the D-glucose stubs, and these chains may, therefore, be removed. Consideration of the results of Larner and coworkers²⁵⁰ on the basis of the revised mechanism of action of the phosphorylase and debranching enzyme system shows that similar results could have been obtained from either of the other two structures, as well as from that proposed by Meyer and coworkers.^{243,244}

Proof of the presence of multiple branching in glycogens and amylopectin comes from an examination of the limit dextrins produced by the

(253) From present knowledge as to the specificity of *beta*-amylase,¹⁴² it is clear that 3 (not 2) maltose residues would have been released from the Cori-Larner ϕ -dextrin.

(253a) D. H. Brown and B. Illingworth, *Proc. Nat. Acad. Sci. U. S.*, **48**, 1783 (1962).

action of purified *alpha*-amylases on these polysaccharides. Thus, Roberts and Whelan¹²² and French^{123,123a} have shown that, in addition to the lower molecular weight *alpha*-limit dextrins that contain a single (1→6)- α -D-glucosidic linkage, higher molecular weight *alpha*-limit dextrins containing 2 or more (1→6)- α -D-glucosidic linkages are also obtained, indicative of multiple branching. Multibranched *alpha*-limit dextrins arise because the branch points are in some places sufficiently close together to prevent *alpha*-amylase action between these branch points. For amylopectin, where the average chain-length is considerably higher than in glycogen, the yield of these dextrins is less, as is apparent from the measurements of the extents of hydrolysis of the two polysaccharides by *alpha*-amylase (see Table II, p. 287).

Some of the limit dextrins from glycogen are so large as to be excluded¹²⁴⁻¹²⁷ from Sephadex G-50. These dextrins have been called macrodextrins, and are, presumably, identifiable with the "dextran-like polysaccharide" isolated by Rozenfel'd from glycogen after *alpha*-amylolysis,²⁵⁴ which, she considered, showed that glycogen is a mixture of two polysaccharides. In this regard, it is worthy of mention that no definitive proof yet exists that the macrodextrins do arise from the glycogen molecules themselves, but, instead, may be present as a separate population of molecules, possibly a byproduct of repeated synthesis and degradation of glycogen. It is, perhaps, significant that the only glycogen reported to yield appreciable amounts of macrodextrins is that from shellfish;²⁵⁵ in higher organisms, such byproducts would be degraded in the lysosomes. The lack of production of macrodextrin from phytoglycogen is, then, consistent with the idea that phytoglycogen, like most plant reserve-polysaccharides, does not undergo turnover, whereas the mammalian polysaccharide does.

A detailed, structural investigation of these unusual dextrins has not yet been made; it might well prove rewarding, particularly if they do, indeed, constitute a true structural feature of the glycogen molecule. Characterization of their structure as being of the Haworth, Staudinger, or Meyer type should be relatively straightforward by use of a combination of the debranching enzymes isoamylase, pullulanase, and glucosidase-transferase, together with *beta*-amylase and purified *Aspergillus niger* glucoamylase. Examination of a related type of polysaccharide (V-glucan) from the envelope of *Vibrio parahemolyticus* by use of two of these enzymes has been reported.²⁵⁶ Debranching with isoamylase and

(254) E. L. Rozenfel'd, *Biokhimiya*, 23, 879 (1958).

(255) M. Schramm, in "Control of Glycogen Metabolism," *Proc. FEBS Meeting*, 4th, Oslo, 1967, W. J. Whelan, ed., Universitetsforlaget, Oslo, 1968, p. 179.

(256) T. Tamura, T. Fujino, H. Miyaji, A. Misaki, and S. Kotani, *Biken's J.*, 12, 231 (1969).

pullulanase yielded mainly maltotriose and maltotetraose, but the authors were unable to reach any conclusions regarding the intramolecular arrangement of the chains.

The formation of *alpha*-amylase-resistant macrodextrins or multi-branched, oligosaccharide *alpha*-limit dextrins after *alpha*-amylolysis of glycogen and amylopectin points to the lack of a high degree of regularity of the lengths of the inner chains of these polysaccharides; this, in turn, must be the result of lack of a high degree of specificity (of the branching enzyme that is involved in their biosynthesis) for the point to which transfer takes place.

It has now been shown that the Meyer model^{243,244} is not a true representation of the structure of glycogen and amylopectin.²⁵⁷ The essential feature of this structure is a regular rebranching so that every B-chain in the molecule carries at least one A-chain. Furthermore, all chains in the Meyer model extend to the periphery of the molecule and, in such a structure, the size of the molecule, as pointed out by French,¹⁴¹ is likely to be limited by the density of the chain ends at the surface becoming so great as to prevent further growth. The availability of the debranching enzyme isoamylase⁴² has permitted a more searching examination of the fine structures of glycogen and amylopectin, and this has shown that the features required by the Meyer model are not present.

The procedure used involved preparation of ϕ -dextrins of both polysaccharides by exhaustive treatment with rabbit-muscle phosphorylase, followed by *beta*-amylolysis to give compounds that have been called ϕ -*beta*-dextrins. In this way, all of the outer (A and B) chains were trimmed to 2 D-glucose residues in length, giving the structure shown in Fig. 4b (see p. 289). It will be readily apparent that, if all B-chains carry A-chains, as required in the structure of Meyer and coworkers,^{243,244} and all of the chains terminate at the surface of the molecule, debranching of the ϕ -*beta*-dextrins should result in a series of maltosyl-terminated oligosaccharides and nothing else, because the specificity of this debranching enzyme is such that it will not remove maltosyl residues. Such dextrins would be resistant to the action of *beta*-amylase, so that *beta*-amylase should be without action on the products obtained by debranching a Meyer-Bernfeld-Fuld ϕ -*beta*-dextrin with isoamylase (see Fig. 11). However, on performing this experiment, it was found that, for amylopectin, 29% of the debranched ϕ -*beta*-dextrin was released as maltose; debranched glycogen ϕ -*beta*-dextrin was degraded²⁵⁷ by *beta*-amylase to the extent of 44%. It was thus clear that some B-chains in the molecules do not bear A-chains. In order to fit the evidence (namely, the presence of approxi-

(257) Z. Gunja-Smith, J. J. Marshall, C. Mercier, E. E. Smith, and W. J. Whelan, *FEBS Lett.*, **12**, 101 (1970).

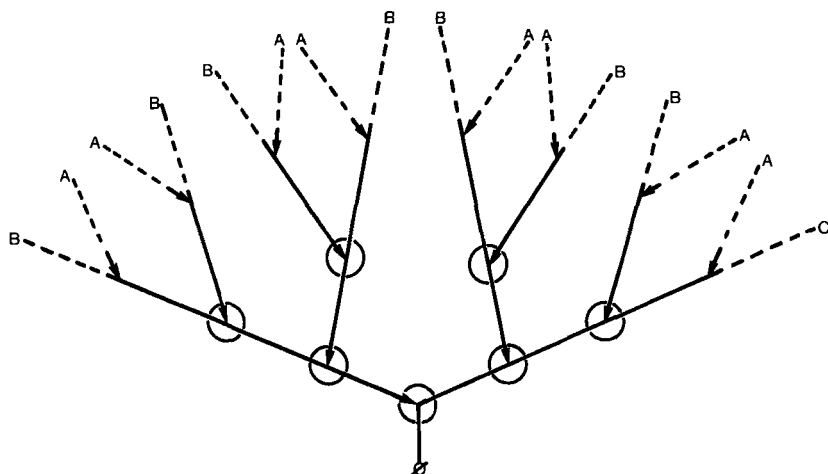


FIG. 11.—Successive Actions of Phosphorylase, *beta*-Amylase, Isoamylase, and *beta*-Amylase on the Meyer Model of Amylopectin and Glycogen. [The symbolism is basically that of Fig. 9, modified to show the action of the various enzymes as follows: - - -, removed by phosphorylase and *beta*-amylase; \ominus , branch points hydrolyzed by isoamylase; and —, immune to *beta*-amylase after isoamylase action.]

mately equal numbers of A- and B-chains in a molecule whose ϕ -*beta*-dextrin could be degraded by *beta*-amylase after debranching), it was necessary to postulate that certain B-chains carry only B-chains and not A-chains, and furthermore, that some B-chains do not reach the surface of the molecule. The biosynthetic implications of this conclusion are obvious; it means that, at any stage in the biosynthesis of these macromolecules, any chain (regardless of whether it be an A-, B-, or C-chain) has only a certain finite chance of being further extended by the chain-extending enzyme. This type of mechanism is in direct contrast to that required on the basis of the Meyer model, where *all* chains must be extended until growth of the molecule is complete. Clearly, the former is a much more likely situation.

Thus, the structure shown in Fig. 12a was proposed by Whelan and coworkers²⁵⁷ to represent diagrammatically the structures of glycogen and amylopectin. It may be seen that this model, which has been drawn in the most symmetrical way possible and in which all B-chains carry, on average, 2 other chains (either 2 A-chains or 2 B-chains), accounts for the liberation of maltose after *beta*-amylase treatment of isoamylase-debranched ϕ -*beta*-dextrins, this maltose coming from B-chains that carry only other B-chains (see Fig. 12b). Unlike the Meyer model, wherein chains do not terminate within the molecule, this feature is characteristic of the new structure. The result is that the molecular size should not be

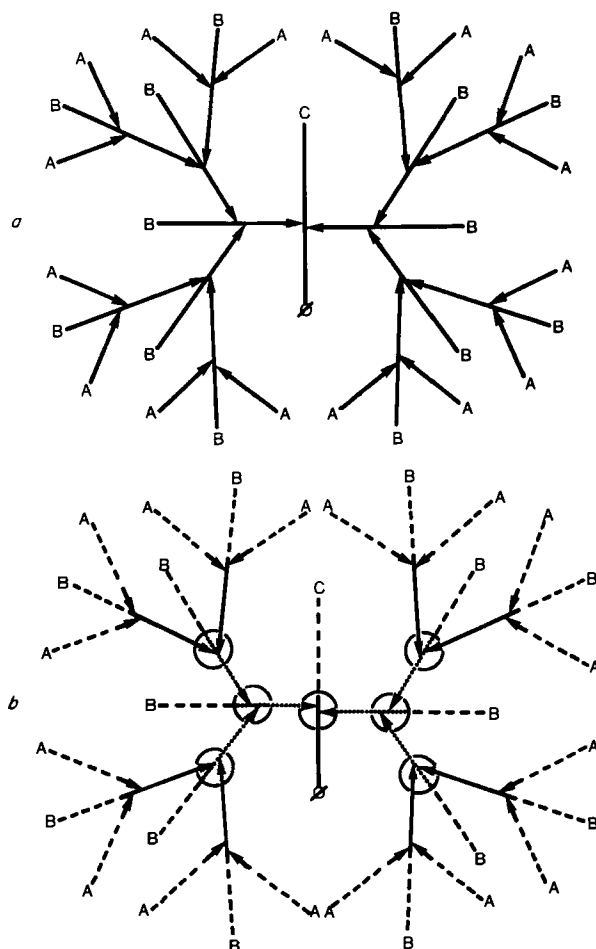


FIG. 12.—(a) Structure Proposed for Glycogen and Amylopectin by Whelan and Coworkers.²⁵⁷ [The symbols are as given in Fig. 9.] (b) Successive Actions of Phosphorylase, β -Amylase, Isoamylase, and β -Amylase on the Whelan Model. [The action of the various enzymes is represented as follows: - - -, removed by phosphorylase and β -amylase; \ominus , branch points hydrolyzed by isoamylase; —, immune to β -amylase after isoamylase action;, removed by β -amylase after isoamylase action.]

limited by the concentration of chain ends at the surface (compare Ref. 141) and that some other factor must determine when growth of the molecules is terminated.

Although the possible presence of sterically inaccessible "buried" B-chains is, therefore, now recognized, the question of "buried" A-chains

remains open. In the molecule as drawn in Fig. 12a, there are no buried A-chains; that is, all are shown as being at the surface. However, the possibility that some A-chains lie deeper inside the molecule, as suggested by French,¹⁴⁰ must be considered. The possible existence of buried chains does raise the question of the accessibility of the chain ends to the *exo*-acting enzymes, in particular, phosphorylase and *beta*-amylase. It is generally assumed that all A- and outer B-chains are accessible to the action of these enzymes, but the improbability of this being so, when the size ($\sim 200,000$ daltons) of these enzymes is considered, and particularly if some chain-ends are buried, has been mentioned.²⁵⁷ An attempt^{257a} was made to answer the question by using the *exo*-acting amylase from *Pseudomonas stutzeri*¹⁰⁹ acting on the *beta*- and ϕ -dextrins of amylopectin and glycogen. In view of its much smaller molecular size (12,000 daltons), it was considered that this enzyme might be able to reach chains inaccessible to the other, larger, *exo*-acting enzymes. Action of the enzyme on these substrates was found to bring about a rapid release of reducing power that soon reached a limit. It was, therefore, concluded that some nonreducing ends are inaccessible to the larger *exo*-enzymes *beta*-amylase and phosphorylase. It was not, however, possible to decide whether these inaccessible ends are present as A-chains or B-chains.

Whereas the proposed model was drawn to conform with the presence of equal numbers of A- and B-chains, subsequent work^{43,84a} showed that this structure does not truly represent both glycogen and

Enzyme used	Reducing power measured
(a) Using ϕ - <i>beta</i> dextrins	
Isoamylase	B-chains
Pullulanase (amylopectin) or isoamylase + pullulanase (glycogen)	<u>B + A-chains</u>
Difference	A-Chains
(b) Using <i>beta</i> -limit dextrins	
Isoamylase	B + $\frac{1}{2}$ A-chains
Pullulanase (amylopectin) or isoamylase + pullulanase (glycogen)	<u>B + A-chains</u>
Difference	$\frac{1}{2}$ A-chains

In each, simple arithmetic then gives the number of B-chains and, hence, the ratio of A-chains: B-chains.

Scheme 2.—How Measurements of the Reducing Power Liberated by the Action of Debranching Enzymes on ϕ -*beta* and *beta*-Limit Dextrins of Glycogen and Amylopectin May be Used to Determine the Relative Numbers of A-Chains and B-Chains in These Polysaccharides.

(257a) J. J. Marshall, unpublished work (1971).

amylopectin. Use of isoamylase, together with the other debranching enzyme pullulanase, facilitated the first accurate determination of the relative numbers of A- and B-chains in both polysaccharides. This problem could be approached in either of two ways, by working with the ϕ -*beta*-dextrins of these polysaccharides, or with the *beta*-amylase limit-dextrins (see Scheme 2). For ϕ -*beta*-dextrins, debranching with isoamylase results in a liberation of reducing power corresponding to the number of B-chains present; the A-chains, which are all maltose residues, are not removed. Subsequent treatment with pullulanase can be used to measure the A-chain content. For *beta*-limit dextrins, half of the A-chains are maltotriose groups and these are removed by treatment with isoamylase. Hence, debranching with pullulanase measures only half of the A-chains. A series of glycogens and amylopectins was examined in this way; the results are shown in Table IV. For this work, the much more readily prepared *beta*-limit dextrins were used.

From a consideration of these results, it is clear that glycogens and

TABLE IV
Determination of Ratios of A- and B-Chains in Samples of Glycogen and Amylopectin^{a,c}

Sample	Reducing power liberated ^a		Number of A-chains per B-chain ^b
	By isoamylase	By isoamylase + pullulanase	
<i>Glycogens</i>			
<i>Ascaris lumbricoides</i>	53.5	66.2	0.6
Shellfish	85.7	110.0	0.8
Phytoglycogen	47.9	62.4	0.9
<i>Trichomonas foetus</i>	52.9	70.5	1.0
Rabbit liver	38.4	51.7	1.1
Human muscle	97.7	131.8	1.1
Cat liver	47.3	65.0	1.2
<i>Amylopectins</i>			
Wheat	73.8	105.2	1.5
Rice	63.0	89.5	1.5
Maize	69.4	101.0	1.7
Potato	46.6	70.0	2.0
Sweet potato	32.8	49.2	2.0
Sweet corn	62.4	95.8	2.3
Waxy maize	56.7	88.9	2.6
Waxy sorghum	18.9	29.6	2.6

^a As μ g of glucose. ^b This is $2(\text{reducing power liberated by isoamylase} + \text{pullulanase} - \text{reducing power liberated by isoamylase}) / [2(\text{reducing power liberated by isoamylase}) - \text{reducing power liberated by isoamylase} + \text{pullulanase}]$.

amylopectins differ in degree of multiple branching. As the A:B-chain ratio for glycogens is (on average) 1:1, whereas that of amylopectins averages 2:1, this ratio shows that, in glycogens, every B-chain carries 2 other chains. In amylopectins, every B-chain must carry, on average, 3 other chains. This is exactly what might be expected from a consideration of the chain lengths of these polysaccharides; that is, because the chains of amylopectin are longer, there is more chance of their carrying a larger number of substituent chains. In addition, the greater degree of multiple branching in amylopectin explains the result obtained earlier as to the amounts of maltose liberated from isoamylase-debranched ϕ -*beta*-dextrins by *beta*-amylase. The higher proportion of A-chains in amylopectin than in glycogen accounts for the lower yield of maltose (29%) from amylopectin ϕ -*beta*-dextrin²⁵⁷ than from glycogen ϕ -*beta*-dextrin (44%). Clearly, the structure depicted in Fig. 12a is, therefore, as drawn, strictly only representative of glycogens; modification to show a higher degree of substitution on the B-chains would be necessary for it to be applicable to amylopectins.

As well as there being, on average, a clear difference between glycogens and amylopectins, it may be seen that there is also a wide variation between individual members of both families of polysaccharides, such that, at the higher extreme of the range for glycogens and the lower extreme for amylopectins, the analyses almost overlap. A similar situation exists with regard to the *beta*-amylolysis limits and chain lengths of these two types of polysaccharide. The ease of this method of measuring the A:B-chain ratio is such as to make it likely to become routine in the characterization of an amylopectin or glycogen, along with other parameters, such as chain length and *alpha*- and *beta*-amylolysis limits. Although the procedure does not take account of the presence of "buried" A-chains, which may not be trimmed to maltose or maltotriose (and therefore counted as B-chains), this is unlikely to make a significant difference to the numerical values obtained, and will not alter the most significant conclusion, namely, that the degree of multiple branching is significantly higher in amylopectin than in glycogen. The biosynthetic implication of this conclusion is that starch cannot be synthesized from a glycogen precursor, as has been proposed by Erlander.²⁵⁸⁻²⁶⁰ Although Erlander's theory for the biosynthesis of the starch components has generally been regarded as unlikely, all evidence against it has hitherto been of a *negative* nature (discussed in Ref. 261). The demonstration

(258) S. R. Erlander, *Enzymologia*, **19**, 273 (1958).

(259) S. R. Erlander, *Stärke*, **22**, 352 (1970).

(260) S. R. Erlander, *Stärke*, **22**, 393 (1970).

(261) D. J. Manners, *Advan. Carbohyd. Chem.*, **17**, 371 (1962).

that amylopectin is more multiply branched than glycogen, however, shows *positively* that biosynthesis of the former polysaccharide cannot involve debranching of a glycogen precursor.

Previous calculations of A:B-chain ratios²⁶² have largely been based on the wrongly assumed action of phosphorylase on the branched polysaccharides, and are therefore inaccurate; this can be seen by comparing the results shown in Table IV with those in Table V. Another study²⁶³ of a series of glycogens, based on the method first applied by Peat and coworkers^{138,247} for the examination of multiple branching in waxy-maize starch by use of R-enzyme, employed glycogen *beta*-limit dextrins and pullulanase, followed by quantitation of the maltose and maltotriose liberated. The results obtained were not definitive, because the authors assumed, but did not prove, that debranching of all A-chains had taken place. Pullulanase brings about total debranching of amylopectin *beta*-dextrin, but glycogen *beta*-dextrin, in contrast, is far from being completely debranched. No evidence exists to suggest that the obvious inability of the enzyme to attack certain chains in glycogen *beta*-dextrin is confined to the B-chains. In addition, the effect of the *alpha*-amylase present in the pullulanase used²⁶⁴ is difficult to assess.

TABLE V

Ratios of A- and B-Chains in Samples of Glycogen and Amylopectin, Determined from Measurements of $\overline{c.l.}$, *beta*-Amylolysis Limits, and Phosphorolysis Limits^a

<i>Polysaccharide</i>	<i>Number of A-chains per B-chain</i>
<i>Glycogens</i>	
Rabbit liver	0.3; 0.3; 0.4; 0.7; ^b 0.9
Cat liver	0.5; 0.5
Human muscle	0.6
Shellfish (<i>Mytilus edulis</i>)	0.4; 0.7
<i>Ascaris lumbricoides</i>	0.5
<i>Amylopectins</i>	
Waxy maize	0.6
Waxy sorghum	0.3
Maize	0.3 ^b
Wheat	0.3 ^b

^a Experimental results of Liddle and Manners,²⁶² except where indicated. ^b Calculated by Liddle and Manners²⁶² from the experimental results of Larner and coworkers.²⁵⁰

(262) A. M. Liddle and D. J. Manners, *J. Chem. Soc.*, 4708 (1957).

(263) G. N. Bathgate and D. J. Manners, *Biochem. J.*, **101**, 3c (1966).

(264) G. N. Bathgate, personal communication (1970).

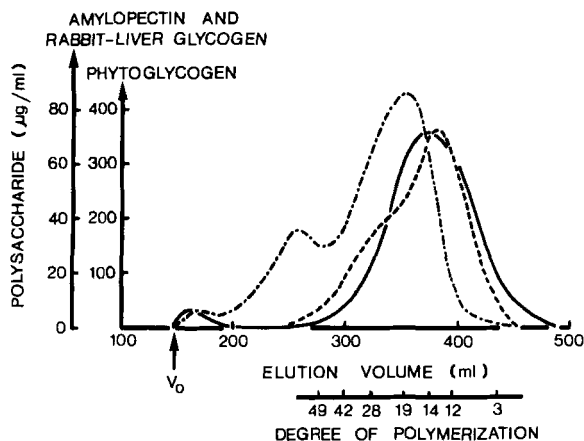


FIG. 13.—Sephadex G-50 Fractionation²⁶⁷ of the Products of Isoamylase-debranching of Rabbit-liver Glycogen (—), Phytoglycogen (- - -), and Amylopectin (- - - -).

Examination of the unit-chain profile of isoamylase-debranched glycogens (see Fig. 13) after fractionation on molecular sieves, such as Sephadex G-50 or Biogel P-10, gave convincing proof of the correctness of the model proposed by Whelan and coworkers,²⁵⁷ rather than the Meyer model. This technique for examination of unit-chain profiles was developed by Lee, Mercier, and Whelan.²⁶⁵ Rabbit-liver and shellfish glycogens give a symmetrical distribution, on a weight basis, around an average that is, approximately, the average chain-length.²⁵⁷ No evidence of any very long chains, which would necessarily be present were the Meyer structure correct, could be detected. A claim by Japanese workers²⁶⁶ that the profiles of debranched glycogens fractionated on a molecular sieve are consistent with the Meyer structure is not supported by any other experimental evidence, and is inconsistent with their earlier results¹⁷⁸ regarding the action of *Pseudomonas* isoamylase on ϕ -beta-dextrins (compare Ref. 257).

Examination of debranched phytoglycogen in this way has shown a clear difference between this glycogen and mammalian glycogens.^{257,267} It had previously been considered that these polysaccharides are identical,²⁶⁸ but the presence of a nonsymmetrical distribution of unit

(265) E. Y. C. Lee, C. Mercier, and W. J. Whelan, *Arch. Biochem. Biophys.*, **125**, 1028 (1968).

(266) H. Akai, K. Yokobayashi, A. Misaki, and T. Harada, *Biochim. Biophys. Acta*, **237**, 422 (1971).

(267) J. J. Marshall, unpublished work.

(268) S. Peat, W. J. Whelan, and J. R. Turvey, *J. Chem. Soc.*, 2317 (1956).

chains in the plant polysaccharide (there being a preponderance of chains on the high molecular weight side of the distribution), showed that this is not so. This observation has still to be interpreted, both biosynthetically and in terms of the fine structure of this glycogen. However, the possibility that the presence of a preponderance of longer chains indicates that this molecule arises from an amylopectin precursor is worthy of consideration.

Examination of the profile of debranched amylopectin (see Fig. 13) shows a distribution of carbohydrate that is inconsistent with the profile that would be expected from a Meyer structure. In this instance, 2 major peaks of carbohydrate are observed.^{268a} The first peak, at d.p. ~ 40 , is always present as the minor component; the second is at a d.p. of ~ 20 D-glucose residues. A Meyer type of structure would require there to be a continuous distribution of chain lengths, ranging from a high to a low d.p., but would not result in a separate peak of higher molecular weight material such as the peak at d.p. 40. As yet, definitive evidence that both fractions arise from the same polysaccharide is lacking. It is possible that only the lower molecular weight peak comes from material that would be regarded as amylopectin, with a structure similar to that proposed.^{84a,257} The other peak could arise from a component, intermediate between amylose and amylopectin, that has been shown to be present in some starches.^{269,270} This problem is clearly worthy of investigation.

Further proof that neither amylopectin nor glycogen conforms to the Meyer structure has come from an examination of the distribution of C-chain lengths in these polysaccharides.^{271,272} This study was performed by labelling the reducing-end groups of these chains by use of tritiated sodium borohydride, or²⁷³ with cyanide- ^{14}C , followed by debranching with isoamylase, and fractionation of the unit chains on Sephadex. On the basis of the Meyer model, the C-chain is, necessarily, the longest chain in the molecule, and the radioactivity would appear in the highest molecular weight fractions; this was, clearly, not so (see Fig. 14).

A liver glycogen isolated from subjects having type IV glycogenosis

(268a) When amylopectin is debranched with pullulanase, a third peak of carbohydrate is often seen at the void volume of the column (compare Fig. 15). Its presence or absence depends on the physical treatment of the starch before debranching,^{268b} and it is considered to consist of a small amount of incompletely debranched material.

(268b) W. J. Whelan and G. Wöber, unpublished work.

(269) A. S. Perlin, *Can. J. Chem.*, **36**, 810 (1958).

(270) R. L. Whistler and W. M. Doane, *Cereal Chem.*, **38**, 251 (1961).

(271) G. Wöber and W. J. Whelan, *Fed. Proc.*, **30**, 1064 (1971).

(272) W. J. Whelan and G. Wöber, in preparation.

(273) J. D. Moyer and H. S. Isbell, *Anal. Chem.*, **30**, 1975 (1958).

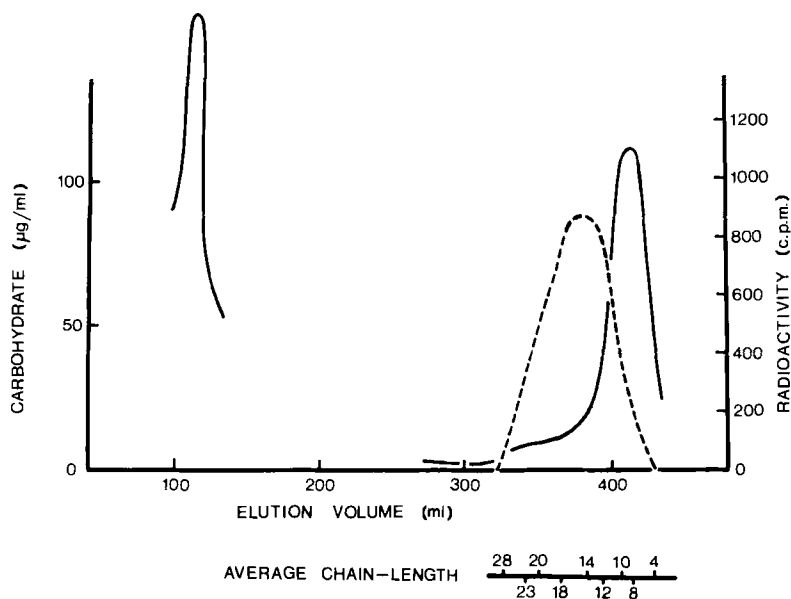


FIG. 14.—Distribution of Carbohydrate (---) and Radioactivity (—) After Debranching, with Isoamylase, of Shellfish Glycogen Reduced with Sodium Borotritide.²⁷²

was originally considered to be identical with amylopectin;²⁷⁴⁻²⁷⁷ hence, the name “amylopectinosis” for this condition. Although, superficially, the properties of the two polysaccharides are almost identical (see Table VI), a more detailed examination of type IV, storage-disease glycogen²⁷⁸ showed it to have a structure quite different from that of waxy-maize amylopectin. After debranching with pullulanase, fractionation on Sephadex showed the type IV glycogen to have a unit-chain profile distinct from that of amylopectin (see Fig. 15). The difference between the type IV polysaccharide and plant amylopectin was also emphasized by the far-from-complete debranching of the former with pullulanase, showing that some characteristics of a glycogen type of polysaccharide were present. Evidence against the presence of two distinct types of polysaccharides, one a normal glycogen, came from pullulanase-debranch-

(274) D. H. Andersen, *Lab. Invest.*, **5**, 11 (1956).

(275) J. B. Sidbury, J. Mason, W. B. Burns, and B. H. Ruebner, *Bull. Johns Hopkins Hosp.*, **111**, 157 (1962).

(276) B. I. Brown and D. H. Brown, *Proc. Nat. Acad. Sci. U. S.*, **56**, 725 (1966).

(277) L. W. J. Holleman, J. A. van der Haar, and G. A. M. Vaan, *Lab. Invest.*, **15**, 357 (1966).

(278) C. Mercier and W. J. Whelan, *Eur. J. Biochem.*, **16**, 579 (1970).

TABLE VI
Properties of Human-liver Glycogen, Type IV Glycogen, and Waxy-maize Amylopectin^{27a}

<i>Polysaccharide</i>	<i>Blue value</i>	λ_{\max} (nm)	$\overline{c.l.}$	$\overline{o.c.l.}^a$	$\overline{i.c.l.}^a$	beta-Amylolysis (%)		(1→6)- α -D-Glucosidic linkages hydrolyzed (%)
						<i>Before debranching</i>	<i>After debranching</i>	
Human-liver glycogen	0.008	475	11.8	7.7	3.1	47	47	0
Type IV glycogen	0.140	525	21.7	14.1	6.6	57	80	43
Waxy-maize amylopectin	0.117	540	21.8	15.0	5.8	60	98	95

^a O.c.l. = outer-chain length; i.c.l. = inner-chain length.

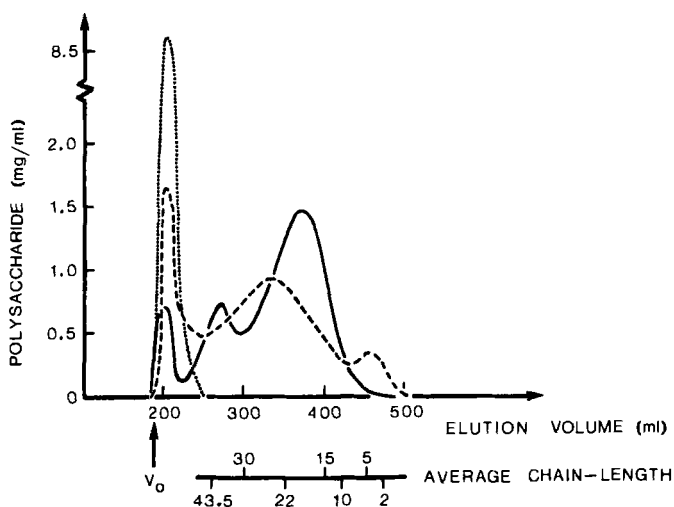


FIG. 15.—Elution Patterns,²⁷⁸ from Sephadex G-50, of Polysaccharides Debranched by Pullulanase. [—, Human-liver glycogen; ---, type IV storage-disease glycogen; and — · —, waxy-maize amylopectin.]

ing of the *beta*-limit dextrin of the type IV polysaccharide, followed by *beta*-amylolysis; this resulted in “103%” conversion of the *beta*-limit dextrin into maltose. The same treatment of normal glycogen *beta*-limit dextrin gave only 42.5% of maltose, and at least four such treatments are necessary to achieve complete degradation, because pullulanase can only debranch the outermost chains in *beta*-limit dextrans of glycogen.

This work also demonstrated most convincingly that the proposal²⁷⁹ that the type IV glycogen is merely a normal glycogen having extended outer chains is incorrect. If type IV glycogen had such a structure, the *beta*-limit dextrans of the normal and type IV glycogens would be identical, which they clearly are not.

d. Routine Characterization of Amylaceous Polysaccharides.—In addition to their use in fundamental structural studies as already described, enzymic methods are widely used routinely for characterization of polysaccharides of the amylose, amylopectin, or glycogen types. Enzymic methods have made this work so facile (it can be done with as little as 5 mg of material and in less than 24 hr) that the more time-consuming and less definitive, nonenzymic methods are now seldom used.

(279) B. I. Brown and D. H. Brown, in “Carbohydrate Metabolism and its Disorders,” F. Dickens, P. J. Randle, and W. J. Whelan, eds., Academic Press, New York, 1968, Vol. 2, p. 140.

The facility of the enzymic methods is evidenced by the large number of publications coming from laboratories specializing in routine analytical studies on polysaccharides of this type,²⁸⁰⁻²⁸⁶ as well as from our own work (see, for example, Ref. 287). Although enzymic methods, by themselves, are generally completely definitive, additional evidence, such as that from iodine-staining measurements, may in some instances be useful.²⁸⁷

Table II (see p. 287) summarizes the measurements that should be made, and the results to be expected, for the three polysaccharides amylose, amylopectin, and glycogen. One of the most important measurements, and the one that all the others depend on, is the total content of (1→4)- α -D-glucan. Such measurements are now made by specific hydrolysis into D-glucose by glucoamylase plus *alpha*-amylase (see Section III, p. 280). In this way, the (1→4)- α -D-glucan content is measured, and the effect of any contaminating polysaccharides is eliminated. In addition, by comparison of the results with the value for total carbohydrate (as measured, for example, by the phenol-sulfuric acid method,²⁸⁸ or by acid hydrolysis and measurement of reducing power), a reliable estimate of the degree of purity is obtained. The figures for *alpha*-amylolysis, *beta*-amylolysis, phosphorolysis, and degradation by pure glucoamylase and by pullulanase plus *beta*-amylase are self-explanatory, and need no further comment.

One of the most significant items of information, particularly for glycogens and amylopectins, comes from a comparison of the effects of isoamylase and pullulanase; and it may be used to classify, very readily, a polysaccharide as an amylopectin or a glycogen. It is now known that carefully isolated glycogens, undegraded during extraction, are virtually unattacked by pullulanase,^{24,174,287} but are completely debranched by isoamylase.⁴² On the other hand, amylopectins are almost completely debranched by both debranching enzymes.^{24,41,42}

Determination of the $\overline{c.l.}$, routinely performed at one time by periodate

(280) C. T. Greenwood and J. Thomson, *J. Inst. Brewing*, **65**, 346 (1959).

(281) C. T. Greenwood, *Stärke*, **12**, 169 (1960).

(282) C. T. Greenwood and J. Thomson, *J. Chem. Soc.*, 222 (1962).

(283) O. Kjøberg, D. J. Manners, and R. A. Lawrie, *Biochem. J.*, **87**, 351 (1963).

(284) D. J. Bell and D. J. Manners, *J. Chem. Soc.*, 3641 (1952).

(285) O. Kjøberg and D. J. Manners, *J. Chem. Soc.*, 4596 (1962).

(286) O. Kjøberg, D. J. Manners, and A. Wright, *Comp. Biochem. Physiol.*, **8**, 353 (1963).

(287) J. J. Marshall and F. R. Rickson, *Carbohydr. Res.*, **28**, 31 (1973).

(288) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

oxidation,²⁸⁹ a relatively time-consuming procedure, has been considerably simplified by the availability of enzymic methods. The merits and disadvantages of these various methods are worthy of discussion.

(i) **Phosphorylase plus "Amylo-(1→6)-glucosidase."**—This is the original, enzymic, chain-length method, developed by Cori and co-workers.²⁹⁰ It depends on the fact that simultaneous action of phosphorylase and the debranching enzyme results in complete degradation to α -D-glucopyranosyl phosphate and D-glucose. The D-glucose is liberated in the amount of one molecule per chain; hence, from the analyses for D-glucose and for α -D-glucosyl phosphate, the chain length ($\overline{\text{c.l.}}$) can be calculated from the following equation.

$$\overline{\text{c.l.}} = \frac{\text{moles of D-glucose} + \text{moles of } \alpha\text{-D-glucosyl phosphate}}{\text{moles of D-glucose}}$$

This procedure is not subject to any of the criticisms resulting from incorrect assumptions regarding the mechanism of debranching or the structure of the phosphorylase limit-dextrin discussed previously, and it has been used routinely with successful results. A deterrent to the use of the procedure is that it requires two purified enzymes, both of which are rather difficult to obtain (particularly, free from branching enzyme, which would interfere with the results). Carter and Lee^{290a} used the same method with yeast glucosidase-transferase instead of muscle glucosidase-transferase.

(ii) ***alpha*-Amylase.**—Measurement of the extent of *alpha*-amylolysis of glycogens may be used for determination of $\overline{\text{c.l.}}$ This method²⁹¹ is not absolute, but depends on an empirical relationship between the extent of *alpha*-amylolysis and the $\overline{\text{c.l.}}$ in several glycogens whose $\overline{\text{c.l.}}$ values have been established by other methods. In view of the low percentage of inter-chain linkages, this method cannot be used directly for amylopectins, although it can be used for their *beta*-dextrins and, knowing the *beta*-amylolysis limit, the $\overline{\text{c.l.}}$ of the parent polysaccharide can then be calculated. This method has not been extensively used.

(iii) **Pullulanase plus *beta*-Amylase.**—The advantage of this method⁹⁹ is that both enzymes needed are readily available. The method depends on the fact that, statistically, the unit chains are present as odd-

(289) D. M. W. Anderson, C. T. Greenwood, and E. L. Hirst, *J. Chem. Soc.*, 225 (1955).

(290) B. Illingworth, J. Larner, and G. T. Cori, *J. Biol. Chem.*, **199**, 631 (1952).

(290a) J. H. Carter and E. Y. C. Lee, *Anal. Biochem.*, **39**, 373 (1971).

(291) D. J. Manners and A. Wright, *J. Chem. Soc.*, 1597 (1962).

numbered and even-numbered chains in equal amounts. Degradation by *beta*-amylase and pullulanase (the latter to remove the branch points) results in degradation of the even-numbered chains from the reducing end, to maltose plus a single maltotriose residue. At the high concentration of *beta*-amylase used, the maltotriose is broken down to D-glucose and maltose. Hence, measurement of the D-glucose after the degradation is complete can be used to give the chain length, by use of the following equation.

$$\overline{\text{c.l.}} = \frac{\text{total polysacch. (as D-glucose, determined by using glucoamylase)}}{2 \times (\text{the D-glucose liberated by } \beta\text{-amylase} + \text{pullulanase})}$$

The method gave results in good agreement with those afforded by other methods, but it has since been criticized,²⁹² the bases for the criticism being the possible interference by the reduced glutathione used to stabilize the *beta*-amylase¹³⁷ and the possible effect of maltase impurity in the latter enzyme.²⁹ However, the availability of preparations of maltase-free *beta*-amylase¹³⁵ (see Section IV,1,b; p. 288) showing no instability has rendered these criticisms no longer viable.

(iv) **Glucosidase-Transferase plus *beta*-Amylase.**—This method depends on the ability of glucosidase-transferase and *beta*-amylase, used together, to degrade amylopectins and glycogens almost completely to maltose and D-glucose, the latter coming from the potential reducing-group involved in each branch-point. The mechanism of the degradation has been discussed at length by Carter and Lee,^{290a} and will not be repeated here. After joint action of the enzymes, measurement of the maltose and D-glucose released into the digests is used to calculate the average chain-length by means of the following equation.

$$\overline{\text{c.l.}} = \frac{2 \times \text{moles of maltose}}{\text{moles of D-glucose}} - 1$$

The amount (unity) subtracted arises because the free D-glucose liberated by the debranching enzyme is also measured by the reducing-value assay for maltose. This procedure depends on the availability of purified glucosidase-transferase, and this is the main drawback to its application. However, the method has given values in good agreement with those afforded by other methods.^{290a}

(v) **Direct Debranching by Isoamylase.**²⁹³—This is the simplest method yet developed for estimation of the average chain-lengths both of glycogens and amylopectins, and it has only become possible since

(292) W. Banks and C. T. Greenwood, *Arch. Biochem. Biophys.*, **136**, 320 (1970).

(293) Z. Gunja-Smith, J. J. Marshall, and E. E. Smith, *FEBS Lett.*, **13**, 309 (1971).

isoamylase became available.⁴² After debranching of the polysaccharide by isoamylase, the free reducing groups liberated are measured by a reducing-power method. From the value obtained, and the concentration of total polysaccharide, $\overline{\text{c.l.}}$ is obtained directly by use of the following equation.

$$\overline{\text{c.l.}} = \frac{\text{total polysaccharide (as D-glucose measured by glucoamylase)}}{\text{reducing gps. liberated [as D-glucose measured by reduction of Cu(II)]}}$$

The advantages of this method are its simplicity and speed, and the fact that only one enzyme (in addition to glucoamylase) is needed and that this enzyme is available commercially,²⁹⁴ free from contaminating carbohydrases. Where the $\overline{\text{c.l.}}$ of a *beta*-limit dextrin is desired, the inability of isoamylase to debranch maltosyl A-chains requires that pullulanase shall also be added (to ensure complete debranching).

Table VII shows, for a number of glycogens and amylopectins, a comparison of the results of $\overline{\text{c.l.}}$ determinations by enzymic methods with those obtained by periodate oxidation; these show the high degree of agreement among the results obtained by the various methods.

For the enzymic determination of the d.p. of linear (1→4)- α -D-glucans, the method developed by Lee and Whelan⁹⁹ [see Section IV,2,d(iii); p. 328] has been used.^{295,296} Addition of pullulanase is unnecessary. Again, the number-average $\overline{\text{c.l.}}$ is obtained from measurements of the amount of D-glucose liberated by *beta*-amylase. The method is reputed to give satisfactory results, being suitable even for values of d.p. as high as 1000–2000. A fundamental criticism of the method, however, is that it relies on the hydrolysis of maltotriose in the presence of levels of maltose that, in such experiments, are extremely high, greatly in excess of the minimum amount needed to cause inhibition of *beta*-amylase.¹⁴⁴ In the absence of any proof that complete degradation of all of the maltotriose from the reducing ends of the odd-numbered, linear chains occurs under these conditions, this method must be regarded with suspicion.

An alternative procedure²⁹⁷ for estimation of the number-average d.p. of (1→4)- α -D-glucans has become apparent from work with partly periodate-oxidized amylose (see p. 266). This method depends on the fact that, when an average of one D-glucose residue per chain has been oxidized, the conversion into D-glucose by *alpha*-amylase-free glucoamylase is only 50%. This is likely to become the preferred method for

(294) British Drug Houses, Ltd., Poole, Dorset, England.

(295) W. Banks and C. T. Greenwood, *Carbohydr. Res.*, **6**, 177 (1968).

(296) As the method was based on a well established principle, first applied by others,⁹⁹ the description "novel" used by the authors was not justified.

(297) J. J. Marshall and W. J. Whelan, unpublished work, 1970.

TABLE VII
Determination of Average Chain-lengths of Glycogens and Amylopectins^a

Polysaccharides	Periodate oxidation	Isoamylase ²⁹³	Method			
			beta-Amylase + gluco- sidase- transferase ^{290a}	Phosphorylase + amylo- (1 → 6)- glucosidase ^{290a, b}	beta-Amylase + pullulanase ⁹⁹	alpha- Amylase ²⁹¹
<i>Glycogens</i>						
Cat liver	13	13	15	14	—	—
Human muscle	11	11	11	12	—	—
Rabbit muscle	13	11	13	14	—	13
<i>Ascaris lumbricoides</i>	11	12	12	13	14	12
Human-liver, type III glycogenosis	6	7	5	5	8	—
Skate liver	13	11	11	12	—	—
<i>Trichomonas foetus</i>	15	14	14	15	—	—
Rabbit liver	14	14	13	15	14	14
Horse diaphragm (pre-rigor)	17	13	14	16	15	16-17
Shellfish	—	10	10	—	—	—
<i>Amylopectins</i>						
Waxy maize	20	20	20	21	20	—
Waxy sorghum	22	20	20	23	20	—
beta-Limit dextrin of potato amylopectin	9-10	10 ^c	10	10	—	—

^a All values have been converted into the nearest whole number of D-glucose residues. All of these values were obtained with the same samples of polysaccharide. ^b Although the results given were obtained by using yeast glucosidase-transferase, the method is the same as that of Illingworth and coworkers,²⁹⁰ who used mammalian glucosidase-transferase. The results of Illingworth and coworkers²⁹⁰ are not shown here, as, for their samples, comparative values obtained by other enzymic methods are not available. ^c This value was obtained by using isoamylase plus pullulanase to bring about hydrolysis of 2-unit A-chains.

TABLE VIII
Enzymic Degradation of Phytoglycogens^{174, 287}

Enzyme(s)	Hydrolysis (%)	
	Sweet-corn phytglycogen	Cecropia peltata phytglycogen
Glucoamylase + <i>alpha</i> -amylase ^a	100	100
<i>beta</i> -Amylase ^b	46.7	52.5
<i>alpha</i> -Amylase ^b	81.5	85.0
Isoamylase ^a	8.7	7.9
Pullulanase ^a	1.3	1.1
Isoamylase + <i>beta</i> -amylase ^{b, c}	109	100

^a Sugars released were determined as glucose equivalents. ^b Sugars released were determined as maltose equivalents. ^c Used consecutively.

estimation of the d.p. values of samples of amylose of high molecular weight. In practice, a solution of the sample of amylose under investigation is oxidized to various small extents by use of a solution of sodium metaperiodate of accurately known concentration, and the degradation by *Rhizopus niveus* glucoamylase is determined at each stage of oxidation. A curve of the type shown in Fig. 3 (see p. 275) may be plotted, from which the extent of oxidation that results in 50% degradation by the enzyme may then be determined, and the d.p. calculated. Theoretically, this method is applicable to any linear polysaccharide for which suitable *exo*-enzymes are available for carrying out the degradation.

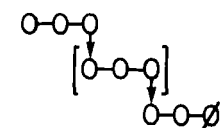
As an illustration of the use of the figures for enzymic degradation in the routine analysis of amylaceous polysaccharides, some work on phytoglycogens may be used. Table VIII shows a comparison of values obtained by enzymic degradation²⁸⁷ of sweet-corn phytglycogen¹⁷⁴ with those for a polysaccharide from ant-foed tissue (*Cecropia peltata*). These figures were regarded as showing that the latter polysaccharide is also a phytglycogen, as indicated by earlier, histochemical studies.²⁹⁸ A unit-chain profile of the debranched polysaccharide on a column of Biogel, together with iodine-stain measurements, supported this conclusion.²⁸⁷

3. Fine Structure of Pullulan

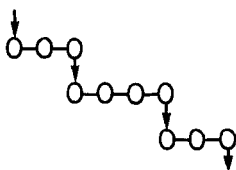
Pullulan, an extracellular glucan produced by the yeast-like fungus *Pullularia pullulans*, generally by growth on sucrose as carbon source, (298) F. R. Rickson, *Science*, **173**, 344 (1971).

was discovered by Wallenfels and coworkers.²⁹⁹ Partial hydrolysis with acid and infrared spectroscopic examination indicated that it contains (1→4)- and (1→6)- α -D-glucosidic linkages.

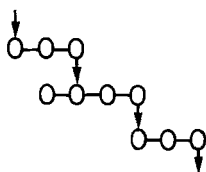
The discovery, shortly thereafter, of the enzyme pullulanase¹⁷¹ from the bacterium *Aerobacter aerogenes* helped considerably in further structural analysis of the polysaccharide. Hydrolysis of pullulan by this enzyme showed that essentially no products containing (1→6)- α -D-glucosidic linkages were formed, the preponderant product being maltotriose.^{171,300} It was thus clear that the enzyme hydrolyzes (1→6)- α -D-glucosidic link-



(a)



(b)



(c)

FIG. 16.—(a) The Gross Structure of Pullulan as Poly[(1→6)- α -maltotriose]. (b) and (c) The Possible Arrangements of the Maltotetraose Residues in the Polysaccharide.

(299) H. Bender, J. Lehmann, and K. Wallenfels, *Biochim. Biophys. Acta*, **36**, 309 (1959).

(300) This is only the case if pullulan is treated (by mild treatment with acid or by boiling) to inactivate an associated α -D-glucosidase; if this is not done, further degradation of the maltotriose occurs.

ages in the substrate. A poly[(1→6)- α -maltotriose] structure (see Fig. 16a) was proposed for the polysaccharide.³⁰¹

Further evidence in favor of this structure, rather than a mixture of starch-type and dextran-type polysaccharides, was its resistance to amylolytic degradation.^{301,302} In addition, periodate oxidation indicated the presence of (1→4)- and (1→6)- α -D-glucosidic linkages in the ratio of 2:1, and methylation analysis yielded the products expected from a structure of the type proposed.^{301,303} Examination of the products of partial hydrolysis of the polysaccharide with acid showed the presence, among other products, of a series of unbranched oligosaccharides containing (1→4)- and (1→6)- α -D-glucosidic linkages.^{171,304} These results were all consistent with a gross structure of the kind shown in Fig. 16a.

A more careful examination of the products of action of pullulanase on pullulan showed the presence of a tetrasaccharide, in addition to maltotriose.³⁰⁵ In the sample examined, the tetrasaccharide was estimated to constitute ~7% of the polysaccharide. The complete hydrolysis of this tetrasaccharide to maltose by sweet-potato *beta*-amylase, as well as optical rotation measurements, characterized it as maltotetraose. As earlier work had suggested that pullulan is a linear molecule, it was, therefore, of interest to ascertain how the maltotetraose units are arranged in the molecule. The two most likely possibilities are illustrated in Fig. 16b,c.

Pullulanase acts in an *endo* fashion,^{55,60} consequently, if the degradation is stopped before completion, oligosaccharides still containing (1→6)- α -D-glucosidic linkages can be isolated. A pure poly[(1→6)- α -maltotriose] would give rise only to maltotriose and higher oligomers containing 6, 9, 12 . . . D-glucose residues and 2, 3, 4 . . . (1→6)- α -D-glucosidic linkages, respectively. However, from the products of partial pullulanolysis, a heptasaccharide fraction was isolated,³⁰⁶ showing that maltotriose and maltotetraose are linked together in the same polysaccharide. After reduction of this heptasaccharide fraction with sodium borohydride, action of pullulanase showed the major, reducing oligosaccharide produced to be maltotetraose. Together with overoxidation with sodium

(301) K. Wallenfels, G. Keilich, G. Bechtler, and D. Freudenberger, *Biochem. Z.*, **341**, 433 (1965).

(302) S. Ueda, K. Fujita, K. Komatsu, and Z. Nakashima, *Appl. Microbiol.*, **11**, 211 (1963).

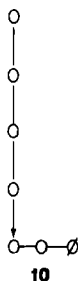
(303) H. O. Bouveng, H. Kiessling, B. Lindberg, and J. McKay, *Acta Chem. Scand.*, **16**, 615 (1962).

(304) H. O. Bouveng, H. Kiessling, B. Lindberg, and J. McKay, *Acta Chem. Scand.*, **17**, 797 (1963).

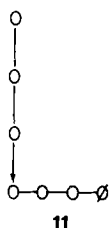
(305) B. J. Catley, J. F. Robyt, and W. J. Whelan, *Biochem. J.*, **100**, 5P (1966).

(306) B. J. Catley and W. J. Whelan, *Arch. Biochem. Biophys.*, **143**, 138 (1971).

metaperiodate, which yielded 2.08 moles of formaldehyde per mole of oligosaccharide, this showed that the heptasaccharide was 6^s- α -malto-tetraosylmaltotriose (10). The presence of a small proportion of malto-

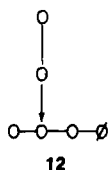


triose after pullulanase debranching of the reduced oligosaccharide indicated the presence of a small proportion of the isomeric heptasaccharide 6^t- α -maltotriosylmaltotetraose³⁰⁷ (11). This result, therefore,



suggested that the maltotetraose units are arranged in a linear molecule through their nonreducing and reducing terminal D-glucose residues.

Further evidence for this arrangement came from a study of the action of the glucoamylase from *Aspergillus niger*, which sequentially removes single D-glucose residues from nonreducing chain-ends. A model oligosaccharide, namely, 6^s- α -maltosylmaltotetraose (12), was rapidly con-



(307) That these two heptasaccharides were not produced in equal amounts, as might be expected on a purely statistical basis, is consistent with the known specificity of pullulanase.⁴¹

verted into 6³- α -D-glucosylmaltotriose (7; see p. 299) and D-glucose, showing that the enzyme has the ability to act rapidly on terminal (1 \rightarrow 4)- α -D-glucosidic linkages attached to D-glucose residues involved in branch points, without prior hydrolysis of the (1 \rightarrow 6)- α -D-glucosidic linkage, which occurs only very slowly. It was argued that, were the maltotetraose in pullulan linked other than through its ends (as shown in Fig. 16c, for example), this would mean that the polysaccharide contains D-glucose units readily accessible to the action of this *exo*-enzyme, and this would be made evident by an initial, rapid release of D-glucose by the enzyme, followed by the relatively slow release of D-glucose from the resulting poly[(1 \rightarrow 6)- α -maltotriose]. When the experiment was performed, there was a uniform rate of release of D-glucose up to 20% hydrolysis of the polysaccharide, confirming the linkage of the tetrasaccharide through both of its terminal, D-glucose residues, and the linear nature of the polysaccharide.³⁰⁶

The specificity of crystalline, salivary *alpha*-amylase is such that it should not act on a poly[(1 \rightarrow 6)- α -maltotriose]. However, action on pullulan results in a limited extent of hydrolysis^{302,306} that could be attributed to the hydrolysis of the maltotetraose residues already shown to be present. Were this interpretation correct, debranching of *alpha*-amylase-treated pullulan would not be expected to yield any maltotetraose; this was confirmed,³⁰⁶ the expected product, 6³- α -D-glucosylmaltotriose (7), being obtained instead (see Fig. 17). It was further concluded that the claim of Wallenfels and coworkers³⁰¹ that the tetrasaccharide component of pullulan contains a (1 \rightarrow 6)- α -D-glucosidic linkage, and arises solely from chain ends, was the result of the action of *alpha*-amylase on the maltotetraose component of the polysaccharide during growth of the organism. Evidence in support of this conclusion was obtained by comparison of pullulan isolated from old cultures (in which there has presumably been more chance for *alpha*-amylolysis to take place) and cultures grown for a less-protracted period.³⁰⁸ The former had a lower molecular weight, and yielded less maltotetraose on debranching by pullulanase.

It is worthy of mention that, although the term pullulan is often used as though it refers to a single, well defined polysaccharide, this is not strictly true. This conclusion is apparent merely from consideration of the differences between the polysaccharide isolated from the same culture after different periods of growth.³⁰⁸ Ueda and coworkers³⁰² examined the extracellular polysaccharide produced by a number of strains of *Pullularia pullulans*, and noted a considerable variation, between strains, in the

(308) B. J. Catley, *FEBS Lett.*, **10**, 190 (1970).

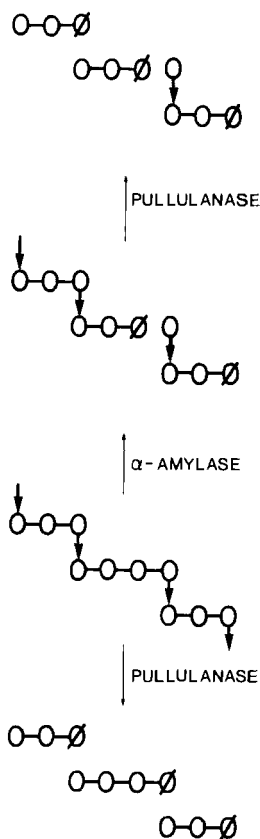


FIG. 17.—Effect of Prior Treatment with α -Amylase on the Products Liberated by Pullulanase from the Maltotetraose Residues in Pullulan.

ratios of the numbers of (1→4)- and (1→6)- α -D-glucosidic linkages. Thus, the term pullulan should no more be taken as referring to a single polysaccharide than, for example, the term glycogen; instead, it is the name for a family of related polysaccharides. Thus, a polysaccharide containing 25% of (1→6)- and 75% of (1→4)- α -D-glucosidic linkages, isolated from deteriorating sugar cane³⁰⁹ (formed from sucrose by *Pullularia pullulans* ?), may also be considered to belong to this family, and the proposed name “sarkaran” cannot therefore be supported. Analysis of this polysaccharide after hydrolysis by pullulanase showed³⁰⁹ maltotriose (48%), maltotetraose (38%), and higher malto-oligosaccharides (13%). As expected from the high percentage of maltotetraose and the

(309) J. Bruijn, *Intern. Sugar J.*, **72**, 195 (1970).

presence of even higher oligosaccharides, the action of *alpha*-amylases (particularly salivary *alpha*-amylase) on this polysaccharide was somewhat greater than on pullulan from other sources.

V. α -D-GLUCANS OTHER THAN STARCH, GLYCOGEN, AND PULLULAN

There are two main types of polysaccharide to be considered under this heading, the first being the dextrans, and the second, α -D-glucans containing solely, or mainly, (1 \rightarrow 3)- α -D-glucosidic linkages.

1. Dextrans

The group of polysaccharides known as dextrans consists of a number of polysaccharides differing rather markedly in molecular constitution.^{310,310a} All polysaccharides considered to be members of this class contain mainly (1 \rightarrow 6)- α -D-glucosidic linkages. Other linkages that may be present are (1 \rightarrow 2)-, or, more commonly, (1 \rightarrow 3)- and (1 \rightarrow 4)- α -D-glucosidic linkages.

Dextranase preparations have been obtained from micro-organisms (bacteria and molds).³¹¹⁻³¹³ Such enzymes are generally *endo*-acting, although there are reports of an *exo*-acting dextranase from a species of *Bacillus*,³¹⁴ and from *Aspergillus niger*.³¹⁵ Generally, production of the enzyme by micro-organisms is stimulated by addition of the substrate to the culture. Dextranases are also present in mammalian tissues;^{316,317} generally, these are *exo*-acting, but they are of little importance, because of their low activity.

An *endo*-dextranase is induced in *Lactobacillus bifidus*³¹¹ by growth on a medium containing an essentially linear dextran (from *Streptococcus bovis*) in which the α -D-glucosidic linkages are almost entirely (1 \rightarrow 6) in nature.³¹⁸ Action of this enzyme on dextrans containing (1 \rightarrow 3)- α -D-glucosidic linkages gave a series of isomalto-oligosaccharides, and other oligosaccharides that were not members of the isomalto-oligosaccharide

(310) W. J. Whelan, *Bull. Soc. Chim. Biol.*, **42**, 1569 (1960).

(310a) R. L. Sidebotham, This Volume, p. 371.

(311) R. W. Bailey and R. T. J. Clarke, *Biochem. J.*, **72**, 49 (1959).

(312) D. H. Hutson and H. Weigel, *Biochem. J.*, **88**, 588 (1963).

(313) E. J. Bourne, D. H. Hutson, and H. Weigel, *Biochem. J.*, **85**, 158 (1962).

(314) L. P. T. M. Zevenhuizen, *Carbohydr. Res.*, **6**, 310 (1968).

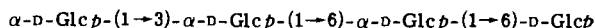
(315) H. M. Tsuchiya, A. R. Jeanes, H. M. Bricker, and C. A. Wilham, *J. Bacteriol.*, **64**, 513 (1952).

(316) E. L. Rozenfel'd, *Biokhimiya*, **21**, 84 (1956).

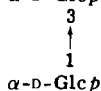
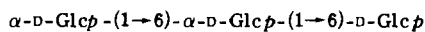
(317) E. L. Rozenfel'd and I. S. Lukomskaya, *Clin. Chim. Acta*, **2**, 105 (1957).

(318) R. W. Bailey, *Biochem. J.*, **71**, 23 (1959).

series.^{319,320} The extents and rates of degradation of the various dextrans tested were dependent on the proportion of (1→3)- α -D-glucosidic linkages present; they were lower as the proportion of such linkages increased.³¹³ These "anomalous" oligosaccharides were not artifacts, as they were not present in digests of linear *Streptococcus bovis* dextran with the enzyme; it was suggested that they contained (1→3)- α -D-glucosidic linkages. The "anomalous" oligosaccharides of lowest molecular weight produced from *Leuconostoc mesenteroides* (Birmingham) dextran were isolated, and it was confirmed that they were not isomalto-oligosaccharides. Nonenzymic characterization showed that the tetrasaccharide fraction, which yielded, among other products, isomaltotriose on acid hydrolysis, was a mixture³²⁰ of two oligosaccharides, namely *O*- α -D-glucopyranosyl-(1→3)-*O*- α -D-glucopyranosyl-(1→6)-*O*- α -D-glucopyranosyl-(1→6)-D-glucopyranose (13) and *O*- α -D-glucopyranosyl-(1→3)-*O*-[α -D-glucopyranosyl-(1→6)]-*O*- α -D-glucopyranosyl-(1→6)-D-glucopyranose (14).



13



14

The pentasaccharide fraction was also considered to be a mixture of oligosaccharides having a similar constitution, consisting of isomalto-tetraose substituted with a (1→3)-linked α -D-glucose residue. This was the first occasion on which the branching region of dextrans had been isolated and characterized. The existence of other, higher molecular weight oligosaccharides, not present as products of enzymic hydrolysis of *Streptococcus bovis* dextran, was taken to indicate that, in places, the branch points are too close together to allow hydrolysis of the (1→6)- α -D-glucosidic linkages between adjacent branch points. Thus, it was concluded that the branch points, of which there is one for every 6 to 7 D-glucose residues, are not regularly, but rather randomly, arranged in the dextran.

Later work³²¹ by the same group utilized two mold-dextranase prepa-

(319) R. W. Bailey, D. H. Hutson, and H. Weigel, *Nature*, **186**, 553 (1960).

(320) R. W. Bailey, D. H. Hutson, and H. Weigel, *Biochem. J.*, **80**, 514 (1961).

(321) E. J. Bourne, D. H. Hutson, and H. Weigel, *Biochem. J.*, **86**, 555 (1963).

rations, also induced by growth on linear dextran.³¹³ These enzymes differed from the bacterial dextranase in giving a greater extent of hydrolysis of dextrans, but were also unable to hydrolyze the linkages constituting branch points.³¹³ Isomaltooligosaccharides having (1→3)-linked α -D-glucose residues were among the products from the action of the bacterial enzyme on *Leuconostoc mesenteroides* dextran;³²¹ these are shown in Fig. 18. There appeared to be slight differences between the specificities of the two mold enzymes, as the smallest oligosaccharide, namely, *O*- α -D-glucopyranosyl-(1→3)-*O*- α -D-glucopyranosyl-(1→6)-*O*- α -D-glucopyranosyl-(1→6)-D-glucopyranose (13), was produced by only one of the enzymes, not the other. However, this result could well have been occasioned by differences in the concentrations of the two enzymes used.

It was considered significant that all of the (1→3)- α -D-linked side-chains in the limit dextrans consist of single D-glucose residues.³¹² The

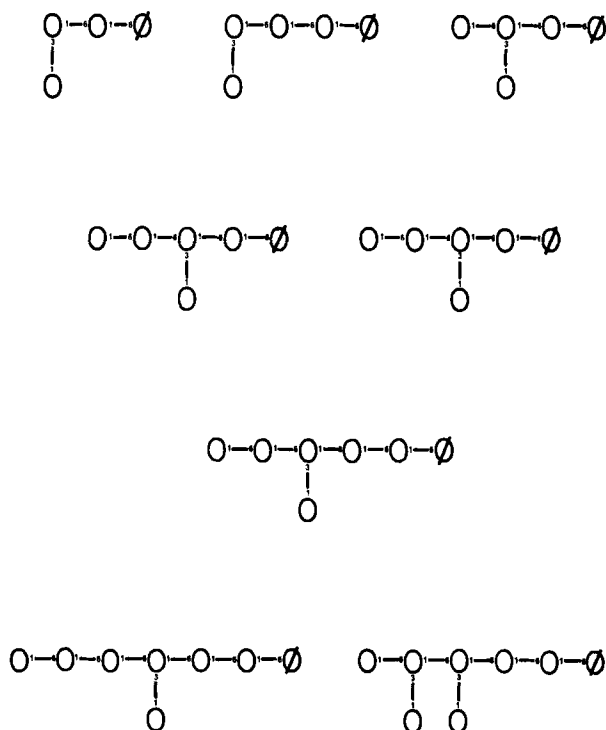


FIG. 18.—Diagrammatic Representation³²¹ of the Oligosaccharides Produced by the Action of Mold Dextranase Preparations on *Leuconostoc mesenteroides* (Birmingham) Dextran. [The symbols are: \bigcirc , α -D-glucosyl residue; \bigcirc , D-glucose residue.]

specificity of the enzyme used was such that, had any side chains in the branched dextran contained 2 or 4 (or more) (1→6)-linked α -D-glucose residues, some (1→3)- α -D-linked isomaltosyl side-chains would also have been present in the products. From the yields of the various branched oligosaccharides, it was calculated that at least 18% of the branches consisted of single D-glucose residues.³¹² Bovey³²² had previously suggested that the majority were of this type. However, nonenzymic studies on two other dextrans have, in contrast, indicated that the side chains are mainly 2 (or more) D-glucose residues long.^{323,323a}

After examination of the *L. mesenteroides* (Birmingham) dextran, the studies were extended to dextrans containing (1→4)- α -D-glucosidic linkages.^{324,325} The same enzymes, namely, bacterial and mold dextranases, were used in conjunction with glucoamylase. Again, branched oligosaccharides were obtained by the action of dextranase; in this instance, they were isomaltose homologs carrying single D-glucose residues joined by (1→4)- α -D-glucosidic linkages.³²⁴ The branch points in the products could be removed by treatment with glucoamylase, yielding the parent isomalto-oligosaccharide. These oligosaccharide products were essentially similar in structure to those obtained from the *L. mesenteroides* dextran, with the exception of the position of branching (to O-4, instead of to O-3 for the D-glucose residues in the isomalto-oligosaccharide). Doubly-branched limit-dextrins were among the products,³²⁴ as with the dextrans containing (1→3)- α -D-linked, branched units.

As well as being able to act on these oligosaccharides, glucoamylase could also act on the parent dextrans containing (1→4)-linked α -D-glucose residues.³²⁵ From NRRL B-1415 dextran, which contains a negligible proportion of (1→3)- α -D-glucosidic linkages and 14.3% of (1→4)- α -D-glucosidic links, 9.4% conversion into D-glucose under the action of glucoamylase occurred. In its action on NRRL B-1416 dextran, which contains 6.9% of (1→4)- and 10.1% of (1→3)- α -D-glucosidic linkages, 5.5% hydrolysis to D-glucose took place. That is, 66 and 80% of the (1→4)- α -D-glucosidic links present in these dextrans took part, respectively. This result showed that at least this proportion of (1→4)-linked α -D-glucose residues is present as nonreducing end-groups. Action of mold dextranase on the glucoamylase-treated dextrans resulted in conversions into isomaltose of 95% (for B-1415) and 59% (for B-1416), in agreement

(322) F. A. Bovey, *J. Polym. Sci.*, **35**, 167 (1959).

(323) D. A. Rees, N. G. Richardson, N. J. Wight, and Sir Edmund Hirst, *Carbohydr. Res.*, **9**, 451 (1969).

(323a) O. Larm, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **20**, 39 (1971).

(324) D. Abbott and H. Weigel, *J. Chem. Soc. (C)*, 821 (1966).

(325) D. Abbott, E. J. Bourne, and H. Weigel, *J. Chem. Soc. (C)*, 827 (1966).

with the result to be expected were these a linear dextran and a dextran containing 10% of (1→3)-linked α -D-glucosyl side-chains, respectively.³²⁵

Thus, the structure emerging from this work is that dextrans are essentially linear, (1→6)-linked α -D-glucans, but that they may be modified by the presence of branch points consisting of (1→3)- or (1→4)-linked α -D-glucosyl side-chains. The enzymic evidence is not inconsistent with the concept that these chains are present mainly as single, α -D-glucosyl side-chains randomly arranged on the main chain, on average, 6 to 7 D-glucose residues apart.

It is unfortunate that none of the work described was performed with purified enzymes. Until this is done, the confidence that can be placed in the foregoing results and in their significance is small. Two electrophoretically homogeneous dextranases have been reported,^{325a,325b} and use of such enzyme preparations may yield more-reliable information. Another matter of concern is the homogeneity of the dextran samples studied. This problem is emphasized by the results of experiments^{325c} on streptococcal dextrans, which showed that fractions containing different proportions of (1→3)- and (1→6)- α -D-glucosidic linkages could be obtained by fractionation with alcohol, indicating the possible presence of more than one polysaccharide. Molecular-sieve chromatography on Sepharose gels should be a useful method for examining the heterogeneity of dextran preparations, and, possibly, even purifying separate constituents thereof (compare Ref. 325d).

It is the opinion of the author that an *exo*-acting dextranase from strains of *Bacillus*³¹⁴ is likely to be a most powerful tool for examining the structures of dextrans, particularly those containing branch points, although this enzyme has not yet been exploited. The enzyme can hydrolyze linear dextrans completely, but has no action on (1→4)- or (1→3)- α -D-glucosidic linkages.³¹⁴ Hence, action on branched dextrans would be expected to be far from complete. As yet, its obvious application in the examination of such polysaccharides, namely to confirm that the side chains are single D-glucose residues, has not been reported. This enzyme may have as important a function in the structural analysis of dextrans as have *beta*-amylase and phosphorylase in investigations of starch and glycogen.¹²

(325a) L. Chalet, A. J. Kempf, R. Harman, E. Kaczka, R. Weston, K. Nollstadt, and F. J. Wolf, *Appl. Microbiol.*, **20**, 421 (1970).

(325b) J. Fukumoto, H. Tsuji, and D. Tsuru, *J. Biochem. (Tokyo)*, **69**, 1113 (1971).

(325c) R. L. Sidebotham, H. Weigel, and W. H. Bowen, *Carbohydr. Res.*, **19**, 151 (1971).

(325d) J. J. Marshall, *J. Chromatogr.*, **77**, 201 (1973).

2. (1→3)- α -D-Glucans

Relatively few glucans containing the (1→3)- α -D-linkage are known, although it has been suggested that they may be of much more widespread occurrence than previously supposed.³²⁶ (1→3)- α -D-Glucans have been isolated from species of *Polyporus*,^{327,327a} from the cell walls of *Aspergillus niger*^{328,329} and *Aspergillus nidulans*,³³⁰ and from several yeasts.³²⁶

(1→3)- α -D-Glucosidic linkages are also found in certain dextrans, sometimes constituting up to 50% of the linkages present. Other glucans containing (1→3)- α -D-glucosidic linkages are isolichenan^{331,332} from Iceland moss [45% of (1→4)- and 55% of (1→3)- α -D-glucosidic linkages] and nigeran [mycodextran; 50% of (1→4)- and 50% of (1→3)- α -D-glucosidic linkages] from the mycelium of certain fungi, including *Aspergillus niger* and *Aspergillus japonicus*.^{333,334} Glucans from mango^{335,336} and from the lichen *Parmelia caperata* (L.) Ach.³³⁷ also contain both (1→3)- and (1→4)- α -D-glucosidic linkages. The amount of enzymic degradation data accumulated for these polysaccharides is thus far somewhat limited, mainly because, although some of these polysaccharides have been known for a considerable time, only recently have enzymes been discovered that will degrade them.

(1→3)- α -D-Glucanase activity was shown to be induced in certain fungi, notably *Trichoderma* and *Penicillium*, by growth on (1→3)- α -D-glucan as the carbon source,³³⁸ and the activity is produced constitutively

- (326) J. S. D. Bacon, D. Jones, V. C. Farmer, and D. M. Webley, *Biochim. Biophys. Acta*, **158**, 313 (1968).
- (327) R. B. Duff, *J. Chem. Soc.*, 2592 (1952).
- (327a) B. J. Ralph and V. J. Bender, *Chem. Ind. (London)*, 1181 (1965).
- (328) I. R. Johnston, *Biochem. J.*, **96**, 651 (1965).
- (329) I. R. Johnston, *Biochem. J.*, **96**, 659 (1965).
- (330) B. J. M. Zonneveld, *Biochim. Biophys. Acta*, **249**, 506 (1971).
- (331) N. B. Chanda, E. L. Hirst, and D. J. Manners, *J. Chem. Soc.*, 1951 (1957).
- (332) S. Peat, W. J. Whelan, J. R. Turvey, and K. Morgan, *J. Chem. Soc.*, 623 (1961).
- (333) S. A. Barker, E. J. Bourne, and M. Stacey, *J. Chem. Soc.*, 3084 (1953).
- (334) S. A. Barker, E. J. Bourne, D. M. O'Mant, and M. Stacey, *J. Chem. Soc.*, 2448 (1957).
- (335) A. Das and C. V. N. Rao, *Tappi*, **47**, 339 (1964).
- (336) A. Das and C. V. N. Rao, *Aust. J. Chem.*, **18**, 845 (1965).
- (337) T. Takeda, Y. Nishikawa, and S. Shibata, *Chem. Pharm. Bull. (Tokyo)*, **18**, 1074 (1970).
- (338) S. Hasegawa, S. Kirkwood, and J. H. Nordin, *Chem. Ind. (London)*, 1033 (1966).

by *Aspergillus nidulans*.³³⁹ The *Trichoderma* enzyme was purified, and examined in some detail.³⁴⁰ As well as (1→3)- α -D-glucans, other glucans containing (1→3)- α -D-glucosidic linkages, namely isolichenan and nigeran, were also degraded. The nature of the linkage cleaved and of the products formed was, however, not reported. This enzyme has found application in the analysis of a galactoglucan containing (1→3)-linked α -D-glucopyranose residues and (1→5)-linked D-galactofuranose residues,³⁴¹ from the cell walls of *Aspergillus niger*. The difficulty in working with this polysaccharide by using such conventional procedures as acid hydrolysis is that the latter procedure results in selective cleavage of the labile, furanose residues, so that D-galactofuranose oligomers, or oligosaccharides containing both D-galactose and D-glucose residues, could not be isolated. However, the use of *Trichoderma endo*-(1→3)- α -D-glucanase has enabled such oligosaccharides to be isolated intact. This enzyme is also likely to be of use in studies on the structure of other glucans containing (1→3)- α -D-glucosidic linkages.

The action of an *endo*-(1→3)- α -D-glucanase from a species of *Streptomyces*³²⁶ on the glucan from *Polyporus betulinus*³²⁷ yielded a series of nigerodextrins similar to that produced by acid hydrolysis of a glucan, characterized nonenzymically as containing (1→3)- α -D-glucosidic linkages, from the cell walls of *Aspergillus niger*.³²⁰ Previous evidence that the α -D-glucose residues in the *Polyporus* glucan are linked (1→3) was not definitive.³²⁷ The same enzyme also aided in the identification of (1→3)- α -D-glucans in the cell walls of certain yeasts.³²⁶ The (1→3)- α -D-glucanase from *Trichoderma* was also claimed to give evidence for the presence of the same type of glucan in the cell wall of the yeast form of *Paracoccidioides brasiliensis*.³⁴² However, as the enzyme preparation used in the latter work contained a large proportion of β -D-glucanase and brought about only 10% hydrolysis of the polysaccharide being examined, giving unidentified products, this claim cannot be considered definitive.

A polysaccharide produced *in vitro* by glucosyltransferases from a species of *Streptococcus* has been characterized as a (1→3)- α -D-glucan by procedures including the action of an enzyme preparation [considered to be a mixture of *endo*- and *exo*-(1→3)- α -D-glucanases] from a species of *Penicillium*; the enzyme hydrolyzed the polysaccharide to D-glucose.^{342a} This result explained the resistance, to the action of dextranase, of the

(339) B. J. M. Zonneveld, *Biochim. Biophys. Acta*, **258**, 541 (1972).

(340) S. Hasegawa, J. H. Nordin, and S. Kirkwood, *J. Biol. Chem.*, **244**, 5460 (1969).

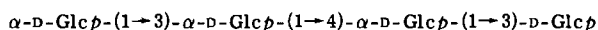
(341) J. H. Nordin, unpublished work, cited in Ref. 340.

(342) F. Kanetsuna and L. M. Carbonell, *J. Bacteriol.*, **101**, 675 (1970).

(342a) B. Cuggenheim, *Helv. Odont. Acta Suppl.* v, **14**, 89 (1970).

polysaccharide produced by this organism, a glucan previously considered to be a dextran.³⁴³

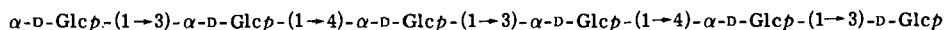
Mycodextranase,²¹ an induced enzyme from *Penicillium melinii* and other fungi, can break down nigeran; it has been of use in confirming the proposed structure of this polysaccharide, and also in studies on the fine structure of isolichenan. The specificity of the enzyme is such that it will hydrolyze only substrates containing both (1→3)- and (1→4)- α -D-glucosidic linkages, the latter linkages being split by the enzyme.^{335,344,345} Nigeran has been assigned a structure consisting of alternate (1→3)- and (1→4)- α -D-linked D-glucose residues on the basis of the results of partial acid hydrolysis, periodate oxidation, and methylation analysis.³³³ The degradation by mycodextranase, which takes place to give complete conversion into nigerose and the tetrasaccharide O- α -D-glucopyranosyl-(1→3)-O- α -D-glucopyranosyl-(1→4)-O- α -D-glucopyranosyl-(1→3)-D-glucopyranose^{21,346} (15), is in agreement with a structure of this type. On



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the basis of the total content of (1→4)- α -D-glucosidic linkages in the products, which requires that (1→4)- α -D-glucosidic linkages be split by the enzyme, as well as the fact that examination of the products showed no evidence for the presence of contiguous (1→3)- or (1→4)- α -D-glucosidic linkages,³⁴⁶ an alternating sequence of the linkages was confirmed.

The smallest known substrate for mycodextranase is the hexasaccharide³⁴⁵ 16, and the enzyme has no action on the tetrasaccharide product



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(15) from nigeran;^{345,346} this shows that the enzyme has high specificity. It has been concluded³⁴⁵ that the minimum specificity requirement is the presence of the pentasaccharide fragment shown in Fig. 19, cleavage taking place at the (1→4)- α -D-glucosidic linkage indicated. Examination of the action of the enzyme on isolichenan³⁴⁵ showed that only a small degree of hydrolysis resulted, to yield oligosaccharides that were not chromatographically mobile. It was thus concluded that alternating

(343) B. Guggenheim and H. E. Schroeder, *Helv. Odont. Acta*, **11**, 131 (1967).

(344) J. H. Nordin, S. Hasegawa, F. Smith, and S. Kirkwood, *Nature*, **210**, 303 (1960).

(345) J. J. Marshall, to be published.

(346) K. K. Tung and J. H. Nordin, *Biochim. Biophys. Acta*, **158**, 154 (1968).

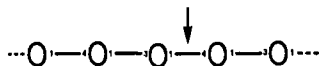


FIG. 19.—Representation of the Minimum Specificity Requirement of Myco-dextranase. [For hydrolysis to occur, a segment of chain containing 5 D-glucose residues joined, as shown, by (1→3)- and (1→4)- α -D-glucosidic linkages is needed. The (1→4)- α -D-linkage marked by an arrow is cleaved.]

sequences of this type are virtually absent from isolichenan. Periodate-oxidation studies have suggested³⁴⁷ the presence in this polysaccharide of sequences of single (or pairs of) (1→3)- α -D-glucosidic linkages flanked on both sides by (1→4)- α -D-glucosidic linkages. As the former arrangement would lead to a significant proportion of mycodextranase-susceptible regions, the enzymic degradation data were regarded as indicating that the latter [pairs of (1→3)-linked α -D-glucose residues] is present in greater excess over single (1→3)-linked α -D-glucose residues flanked by (1→4)- α -D-glucosidic linkages than had been suggested by the results of periodate oxidation.

With iodine, preparations of isolichenan give a blue stain. Formation of the stain can be completely eliminated by treatment with salivary *alpha*-amylase, but only incompletely by *beta*-amylase action.³³² However, it was not possible to ascertain whether the iodine staining-power was attributable to contaminating starch, or to the presence of relatively long sequences of (1→4)-linked α -D-glucose residues in the isolichenan molecules. Subsequent work,³⁴⁵ in which isolichenan was used to test the ability of *alpha*-amylase to cleave D-glucosidic linkages other than (1→4)- α -D (compare the situation with *beta*-glucan hydrolases; see p. 351), has clarified the situation. Treatment of the polysaccharide with *beta*-amylase and pullulanase resulted in complete loss of the iodine-staining power of the preparation, suggesting that the iodine-staining power is due to contaminating starch. Subsequent treatment with *alpha*-amylase gave virtually no further increase in reducing power, showing the absence of any significant proportion of sequences of more than two, contiguous, (1→4)- α -D-glucosidic linkages in isolichenan.

An α -D-glucanase from *Aspergillus oryzae* has been partly purified and has been claimed to be specific for the glucan from mango,^{335,336} which it hydrolyzes to the extent³⁴⁸ of 37%. This polysaccharide, reputedly resistant to *alpha*-amylase action, despite its giving a violet color with iodine, has been assigned a structure consisting of blocks of (1→3)- and (1→4)- α -D-glucosidic linkages, the latter being present in slight excess ($\sim 55\%$). However, in the absence of a detailed characterization of the products of enzymic degradation, the specificity and action pattern of

(347) M. Fleming and D. J. Manners, *Biochem. J.*, **100**, 24P (1966).

(348) A. K. Kundu and S. Manna, *Indian J. Exp. Bot.*, **9**, 75 (1971).

the enzyme and the structure of the polysaccharide must remain in doubt. It is difficult to envisage that the structure is of the type proposed, as this would be expected to be susceptible to *alpha*-amylolysis at the (1→4)-linked regions. Furthermore, the optical rotation ($[\alpha]_D + 166^\circ$) of the polysaccharide differs considerably from that of nigeran ($[\alpha]_D + 272^\circ$; Ref. 332). It is possible that this polysaccharide is a mixture of two components, one of which contains β -D-glucosidic linkages; this could readily be tested by use of appropriate enzymes.

VI. β -D-GLUCANS

β -D-Glucans function as structural components in the cell walls of many plants and micro-organisms. Other glucans of this type are known that act as reserve polysaccharides. Many of the polysaccharides of this group are highly insoluble, making the use of enzymes in their structural analysis difficult, or, sometimes, impossible.

The most important structural applications of the β -D-glucan hydrolases have been in the examination of the mixed-linkage glucans of the lichenan³⁴⁰ and cereal glucan^{350,351} type, and in studies of fungal and yeast cell-wall polysaccharides. Application to the analysis of the fine structures of such polysaccharides as laminaran, in which the majority of the D-glucosidic linkages are of a single type, has been surprisingly small.

Because of the lack of homogeneous, purified, cellulose-degrading enzymes, having a variety of action patterns, that will act on the native, unmodified polysaccharide, it has not yet been possible to investigate the fine structure of cellulose in a manner analogous to that used for amylose. The situation is complicated by the insolubility of the substrate and its resistance to degradation by any single enzyme. A system of enzymes, the function of at least one component of which is at present unclear, is required.^{352,352a} For this reason, it has not yet been possible to examine the fine structure of cellulose enzymically.

1. Enzymes Involved in the Hydrolysis of β -D-Glucosidic Linkages

For the most part, the enzymes hydrolyzing β -D-glucosidic linkages have not been nearly so well characterized as the amylolytic enzymes. The reasons for this are two-fold. Firstly, their substrates tend to be insoluble, for example, cellulose and the (1→3)- β -D-glucans from plants

(349) K. H. Meyer and P. Gürtler, *Helv. Chim. Acta*, **30**, 751 (1947).

(350) I. A. Preece and K. G. MacKenzie, *J. Inst. Brewing*, **58**, 353, 457 (1952).

(351) I. A. Preece and R. Hobkirk, *J. Inst. Brewing*, **59**, 385 (1953).

(352) K. W. King and M. I. Vessal, *Advan. Chem. Ser.*, **95**, 7 (1969).

(352a) D. R. Whitaker, *Enzymes*, **5**, 273 (1971).

(callose³⁵³) and the yeast cell-wall.³⁵⁴ Secondly, the enzymes are often produced as mixtures of enzymes having different action patterns and specificities towards the same substrate, so that purification is difficult. In addition, one of the common media for enzyme purification, *O*-(2-diethylaminoethyl)cellulose, often cannot be used in the purification of some enzymes of this type because it is a substrate for them. However, advantage has, in several instances^{354a,354b,354c} been taken of interaction of β -glucanases with this medium in order to effect purification on the basis of affinity binding.

a. (1 \rightarrow 4)- β -D-Glucanases.^{354d}—Several *endo*-(1 \rightarrow 4)- β -D-glucan hydrolases, mostly from molds, have been purified to a high degree. Thus, Whitaker has reported the isolation of an apparently homogeneous enzyme of this type from *Myrothecium verrucaria*,³⁵⁵ a similar enzyme from *Aspergillus niger* has been purified by Clarke and Stone.^{356,357} A species of *Streptomyces* produces, in large amounts, *endo*-(1 \rightarrow 4)- β -D-glucanase that is relatively pure, at least insofar as freedom from β -D-glucosidase is concerned.³⁵⁸ A (1 \rightarrow 4)- β -D-glucanase of low molecular weight from *Penicillium notatum* has also been purified.³⁵⁹ The patterns of action on cello-oligosaccharides, of the (1 \rightarrow 4)- β -D-glucan hydrolases from *Pseudomonas fluorescens*, have been examined in detail;³⁶⁰ this is one of the few instances where this has been done.

Commercial, (1 \rightarrow 4)- β -D-glucanase (cellulase) preparations are generally grossly impure, and quite unsuitable for structural work. A common source of crude (1 \rightarrow 4)- β -D-glucanase is snail-gut juice,^{361-361b} but it has been shown³⁶² that this enzyme is a minor constituent of the β -D-glucan hydrolase mixture present, so that use of the crude material for

(353) G. Kessler, *Ber. Schweiz. Botan. Ges.*, **68**, 5 (1958).

(354) W. Z. Hassid, M. A. Joslyn, and R. M. McCready, *J. Amer. Chem. Soc.*, **63**, 295 (1941).

(354a) J. J. Marshall, *Anal. Biochem.*, **53**, 191 (1973).

(354b) J. J. Marshall, *J. Chromatogr.*, **76**, 257 (1973).

(354c) J. J. Marshall, *Biochem. Soc. Trans.*, **1**, 198 (1973).

(354d) Often referred to as cellulase, although the enzymes of this type, by themselves, do not generally act on native cellulose.

(355) D. R. Whitaker, *Arch. Biochem. Biophys.*, **43**, 253 (1953).

(356) A. E. Clarke and B. A. Stone, *Biochem. J.*, **96**, 793 (1965).

(357) A. E. Clarke and B. A. Stone, *Biochem. J.*, **96**, 802 (1965).

(358) E. T. Reese, E. Smakula, and A. S. Perlin, *Arch. Biochem. Biophys.*, **85**, 171 (1959).

(359) G. Pettersson, E. B. Cowling, and J. Porath, *Biochim. Biophys. Acta*, **67**, 1 (1963).

(360) K. Yamane, H. Suzuki, and K. Nisizawa, *J. Biochem. (Tokyo)*, **67**, 19 (1970).

(361) M. Holden and M. V. Tracey, *Biochem. J.*, **47**, 407 (1950).

(361a) F. L. Myers and D. H. Northcote, *J. Exp. Biol.*, **35**, 639 (1958).

(361b) F. L. Myers and D. H. Northcote, *Biochem. J.*, **71**, 749 (1959).

structural studies is unwise. A procedure has, however, been developed for the purification of *Helix pomatia* (1→4)-β-D-glucanase.^{362a}

The specificities of (1→4)-β-D-glucanases are often not highly rigid, with the result that their use is not restricted to the structural analysis of polysaccharides of the same type as the natural substrate. Thus, certain enzymes of this type will also degrade (1→4)-β-D-linked xylans^{363,364} and glucomannans.³⁶⁵ In addition, the specificity of these enzymes has not been demonstrated to extend to the nature of the bond split by the enzyme, rather than towards that part of the substrate to which enzyme-substrate binding takes place. By analogy with (1→3)-β-D-glucan hydrolases, it may be that enzyme-substrate binding requires (1→4)-β-D-glycosidic linkages, but that both (1→3)- and (1→4)-β-D-glycosidic linkages can be hydrolyzed (compare Refs. 45-47a).

From a variety of sources have been reported (1→4)-β-D-glucanases that are reputedly cellobiosyl hydrolases acting in a stepwise fashion from the nonreducing chain-end³⁶⁶⁻³⁶⁸ (compare *beta*-amylase). Enzymes of this type are likely to be useful, for example, in the analysis of the oligosaccharides resulting from the degradation of such mixed-linkage glucans as lichenan by *endo*-acting enzymes, and, possibly, also in the analysis of oligosaccharides produced by action of cellulase on glucomannan. However, reports of the identification of cellobiosyl *exo*-hydrolases on the basis of the production of cellobiose from insoluble cellulose must be regarded with some suspicion, because such results may be illusions caused by the physical nature of the substrate.³⁵²

There are few substantiated reports of enzymes of the *exo*-(1→4)-β-D-glucan glucohydrolase type. Such an enzyme would be of great importance, particularly in the analysis of enzymic or nonenzymic degradation-products of polysaccharides containing (1→4)-β-D-glucosidic linkages. One of the most convincing reports of this type of enzyme is that³⁶⁹ of an enzyme from *Stachybotrys atra* which can release D-glucose from (1→4)-β-D-oligoglucosides of d.p. up to 11. Another enzyme of this type is a constituent of the cellulase system of *Trichoderma viride*.³⁷⁰

(362) J. J. Marshall, unpublished work.

(362a) J. J. Marshall, *Comp. Biochem. Physiol.*, **44B**, 981 (1973).

(363) C. T. Bishop and D. R. Whitaker, *Chem. Ind.* (London), 119 (1955).

(364) R. Thomas, *Aust. J. Biol. Sci.*, **9**, 159 (1956).

(365) P. Kooiman, *Enzymologia*, **18**, 371 (1957).

(366) W. O. Storvick and K. W. King, *J. Biol. Chem.*, **235**, 303 (1960).

(367) W. O. Storvick, F. E. Cole, and K. W. King, *Biochemistry*, **2**, 1106 (1963).

(368) Y. W. Han and V. R. Srinivasan, *Appl. Microbiol.*, **16**, 1140 (1968).

(369) G. Youatt, *Aust. J. Biol. Sci.*, **11**, 209 (1958).

(370) L. H. Li, R. M. Flora, and K. W. King, *Arch. Biochem. Biophys.*, **111**, 439 (1965).

b. (1→3)- β -D-Glucanases.³⁷¹—Enzymes of this class have been characterized rather more satisfactorily than many of the other β -D-glucan hydrolases, and it is possible to distinguish several types, differing in specificity.

(i) **Specific (1→3)- β -D-Glucanases.**—These enzymes act only on (1→3)- β -D-glucans and not on the mixed-linkage, (1→3:1→4)- β -D-glucans, such as lichenan and barley glucan. Enzymes of this type from plants and micro-organisms have been purified. Even within the group, there are differences in specificity between some of these enzymes, as shown by the nature of the products formed. Thus, purified enzymes of this type from plant sources (malted barley and other malted cereals) degrade laminaran with production of (1→3)- β -D-linked oligosaccharides but no D-glucose;^{78a,372} the tetrasaccharide laminaratetraose is the smallest substrate for these enzymes. An enzyme from a yeast has a similar specificity.³⁷³ A bacterial enzyme of this type, isolated from a species of *Cytophaga*,³⁷⁴ acts on laminaran, yeast glucan, and pachyman³⁷⁵ with production of oligosaccharides of d.p. 5 and above. In view of its ability to degrade insoluble (1→3)- β -D-glucans³⁷⁶ readily, it should be useful for examining the structures of such polysaccharides not susceptible to degradation by other (1→3)- β -D-glucanases (compare p. 276).

(ii) **Nonspecific (1→3)- β -D-Glucanases.**—The best known example of a nonspecific (1→3)- β -D-glucanase is the enzyme from *Rhizopus arrhizus*,^{44,377} but a similar enzyme is also present in other fungi^{377a} and in malted barley.^{78a} A (1→3)- β -D-glucanase from *Bacillus circulans*³⁷⁸ also has the ability to degrade barley β -D-glucan³⁷⁹ and is, therefore, another member of this group of enzymes. One of the most highly purified and rigorously characterized enzymes of this type has been the subject of work in the author's laboratory; the enzyme, also produced by *Cytophaga*,^{374,376} degrades laminaran, giving, as end products, mainly D-glucose

(371) Enzymes of this type are often referred to as laminaranases.

(372) D. J. Manners and J. J. Marshall, *Phytochemistry*, **12**, 547 (1973).

(373) A. T. H. Abd-El-Al and H. J. Phaff, *Can. J. Microbiol.*, **15**, 697 (1969).

(374) J. J. Marshall, *Carbohydr. Res.*, **26**, 274 (1973).

(375) S. Warsi and W. J. Whelan, *Chem. Ind.* (London), 1573 (1957).

(376) J. J. Marshall and B. A. Buckland, *Abstr. 9th Int. Congr. Biochem., Stockholm*, 104 (1973).

(377) J. J. Marshall, *Biochem. Soc. Trans.*, **1**, 445 (1973).

(377a) G. L. Schneberger and W. W. Luchsinger, *Can. J. Microbiol.*, **13**, 901 (1967).

(378) K. Horikoshi, H. Koffler, and K. Arima, *Biochim. Biophys. Acta*, **73**, 267 (1963).

(379) O. Igarashi, M. Igoshi, and Y. Sakurai, *Agr. Biol. Chem.* (Tokyo), **30**, 1254 (1966).

and laminarabiose; lichenan and barley glucan give products apparently identical with those obtained by the action of the *Rhizopus arrhizus* enzyme.^{45,47,380} The (1→3)-β-D-glucanase from *Cytophaga* was purified by taking advantage of a very strong, but not irreversible, binding of the enzyme to *O*-(2-diethylaminoethyl)cellulose that was presumed to be due to the substrate-like nature of the ion exchanger.^{354a} In this way, the enzyme was obtained in a homogeneous form, by a one-step procedure, directly from an acetone precipitate of a culture filtrate of the organism.^{354a,380} An affinity-chromatographic type of purification of another (1→3)-β-D-glucanase, one of those present in snail juice,³⁶² has been reported^{380a} that employed chromatography on columns of pachyman.

The specificity of this type of enzyme is not directed towards the linkage split by the enzyme. Thus, these enzymes appear to bind to (1→3)-β-D-glucosidic linkages in the substrates, but may split either (1→3)- or (1→4)-β-D-glucosidic linkages,^{45-47a} so that both mixed-linkage glucans and (1→3)-β-D-glucans are hydrolyzed.

(iii) *exo*-(1→3)-β-D-Glucanase.—Several examples of enzymes of this type are known, that having had the most application being from a species of Basidiomycete.^{36,44,381} Similar enzymes have been obtained from *Euglena gracilis*^{382,383} and other organisms.^{384,385} An enzyme of this type from *Sclerotinia libertiana* has been crystallized.³⁸⁶ Some of these enzymes have been quite well characterized, insofar as specificity and properties are concerned; they act on (1→3)-β-D-linked oligosaccharides and polysaccharides in an *exo* fashion, releasing D-glucose from the nonreducing chain-ends. Under certain conditions, however, if a (1→3)-β-D-glucosidic linkage is the penultimate linkage, an *endo* type of hydrolysis also occurs, with liberation of a disaccharide.^{381,386} The particularly attractive feature of enzymes of this type, at least as far as use in structural studies is concerned, is that they do not appear to have the ability to catalyze transglycosylation reactions.³⁸¹

(iv) *Lichenase*.—This term is used to describe an enzyme specific for the hydrolysis of linkages in such mixed-linkage glucans as lichenan

(380) J. J. Marshall, unpublished work.

(380a) D. W. Noble and R. J. Sturgeon, *Biochem. J.*, **110**, 7P (1968).

(381) T. E. Nelson, J. Johnson, E. Jantzen, and S. Kirkwood, *J. Biol. Chem.*, **244**, 5972 (1969).

(382) D. R. Barras and B. A. Stone, *Biochim. Biophys. Acta*, **191**, 329 (1969).

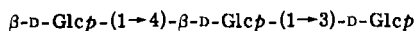
(383) D. R. Barras and B. A. Stone, *Biochim. Biophys. Acta*, **191**, 342 (1969).

(384) A. T. H. Abd-El-Al and H. J. Phaff, *Biochem. J.*, **109**, 347 (1968).

(385) A. Kaji, T. Ohsaki, and O. Yoshihara, *Nippon Nogei Kagaku Kaishi*, **45**, 278 (1971).

(386) J. Ebata and Y. Satomura, *Agr. Biol. Chem. (Tokyo)*, **27**, 478 (1963).

and barley glucan, without any action on (1→4)- or (1→3)- β -D-glucans. The main product is the trisaccharide *O*- β -D-glucopyranosyl-(1→4)-*O*- β -D-glucopyranosyl-(1→3)-D-glucopyranose³⁸⁷ (17). Enzymes of this type



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are produced by bacteria, in particular, by certain species of *Bacillus subtilis*,³⁸⁸ and partial purification has been effected.³⁸⁹ Besides its application in structural studies, the enzyme is of use³⁸⁷ in the preparation of large quantities of the trisaccharide 17.

(v) (1→6)- β -D-Glucanases.—Enzymes of this type are produced constitutively by a number of fungi,^{389a} but growth on media containing a (1→6)- β -D-glucan (such as pustulan³⁹⁰ or lutean³⁹¹) results in increased yields of enzyme. Generally, the culture filtrates contain, in addition, other interfering enzymes. Thus, *Penicillium brefeldianum*^{389a} produces large proportions of (1→6)- β -D-glucanase by growth on pustulan, and also (1→3)- β -D-glucanase and β -D-glucosidase (which must be removed before use). The detailed specificity of fungal (1→6)- β -D-glucanases has not been documented, but they appear to act by an *endo* mechanism. Purification of such enzymes by zone electrophoresis has been described.^{389a} There is also a report of (1→6)- β -D-glucanase in several *Streptomyces*,³⁹² but no information is available on the specificity or action pattern. These enzymes are of potential, rather than demonstrated, application.

(vi) (1→2)- β -D-Glucanase.³⁹³—This enzyme is induced by growth of certain fungi on a suitable carbon source, namely the (1→2)- β -D-glucan from crown-gall tissue.³⁹⁴ Other β -D-glucanases are also produced. In

- (387) E. T. Reese and A. S. Perlin, *Biochem. Biophys. Res. Commun.*, **12**, 194 (1963).
- (388) E. A. Moscatelli, E. A. Ham, and E. L. Rickes, *J. Biol. Chem.*, **236**, 2858 (1961).
- (389) E. L. Rickes, E. A. Ham, E. A. Moscatelli, and W. H. Ott, *Arch. Biochem. Biophys.*, **60**, 371 (1962).
- (389a) E. T. Reese, F. W. Parrish, and M. Mandels, *Can. J. Microbiol.*, **8**, 327 (1962).
- (390) B. Lindberg and J. McPherson, *Acta Chem. Scand.*, **8**, 985 (1954).
- (391) C. G. Anderson, W. N. Haworth, H. Raistrick, and M. Stacey, *Biochem. J.*, **33**, 272 (1939).
- (392) N. Nakamura and O. Tanabe, *Nature*, **200**, 1337 (1963).
- (393) E. T. Reese, F. W. Parrish, and M. Mandels, *Can. J. Microbiol.*, **7**, 309 (1961).
- (394) F. C. McIntire, W. H. Peterson, and A. J. Riker, *J. Biol. Chem.*, **143**, 491 (1942).

view of the rarity of (1→2)- β -D-glucans in Nature, the enzyme is probably of little importance.

(vii) β -D-Glucosidases.—The main application of β -D-glucosidases is in the structural investigation of oligosaccharides arising from the degradation of polysaccharides by other means (enzymic and nonenzymic). These enzymes are, generally, relatively unspecific, or else exist as mixtures whose components may be difficult to separate, possibly even existing as an aggregate. For example, studies on almond emulsin indicated that the glycosidase activities hydrolyzing β -D-glucosides and β -D-galactosides reside in the same enzyme molecule.^{32a} In addition to lack of glycon specificity, the enzymes show a lack of linkage specificity, and (1→1)-, (1→2)-, (1→3)-, and (1→6)-, in addition to (1→4)-, β -D-glycosidic linkages may be cleaved. An important precaution to be taken during the use of these enzymes is against the possibility that artifacts may result from transferase activity in the enzyme preparations or as a result of enzyme-catalyzed, reversion reactions.⁷⁵ Some β -D-glucosidases are not suitable for application in structural work, as they act only on aryl β -D-glucosides,^{395,396} and not on substrates wherein the "aglycon" is also carbohydrate in nature.

2. Applications of β -D-Glucan Hydrolases in the Structural Analysis of β -D-Glucans

a. Cereal β -D-Glucans and Lichenan.—Nonenzymic studies, mainly involving methylation analysis and periodate oxidation, showed that these polysaccharides are essentially unbranched, and contain (1→3)- and (1→4)- β -D-glucosidic linkages in similar proportions.^{331,349,397-401} Although analyses from different laboratories are not in complete agreement as to the relative proportions of the two types of linkage, it is probable that the cereal glucans contain approximately 25%, and lichenan, 35%, of (1→3)- β -D-glucosidic linkages, the remainder being (1→4)- β -D in both. These glucans are readily soluble in hot water.

A study of lichenan by examination of the products of partial hydrolysis with acid established the general features of the polysaccha-

(395) M. A. Jermyn, *Aust. J. Biol. Sci.*, **8**, 541 (1955).

(396) M. A. Jermyn, *Aust. J. Biol. Sci.*, **8**, 577 (1955).

(397) O. Igarashi and Y. Sakurai, *Agr. Biol. Chem.* (Tokyo), **30**, 642 (1966).

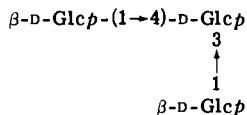
(398) G. O. Aspinall and R. G. Telfer, *J. Chem. Soc.*, 3519 (1954).

(399) L. Acker, W. Diemair, and E. Samhammer, *Z. Lebensmitt.-Untersuch.-Forsch.*, **100**, 180 (1955).

(400) L. Acker, W. Diemair, and E. Samhammer, *Z. Lebensmitt.-Untersuch.-Forsch.*, **102**, 225 (1955).

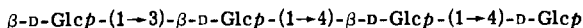
(401) S. Peat, W. J. Whelan, and J. G. Roberts, *J. Chem. Soc.*, 3916 (1957).

ride.⁴⁰¹ In addition to D-glucose, laminarabiose, and cellobiose, three trisaccharides were obtained, one of which was identified as cellotriose; laminaratriose was not observed. Action of almond emulsin on the two separated components of the mixed-linkage trisaccharide fraction yielded, in each instance, only D-glucose and a single disaccharide (in one, laminarabiose, and in the other, cellobiose). On the basis that the other possible trisaccharide from such a polymer (3,4-di-O-β-D-glucopyranosyl-D-glucopyranose, 18) would have yielded two disaccharides

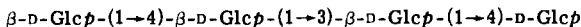


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(laminarabiose and cellobiose), it was concluded that 18 was absent, thus confirming the linear nature of the polysaccharide. Evidence was also obtained for the presence, in the tetrasaccharide fraction from the products of acid hydrolysis, of the tetrasaccharide O-β-D-glucopyranosyl-(1→3)-O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→4)-D-glucopyranose (19) or O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→3)-O-β-D-glucopyranosyl-(1→4)-D-glucopyranose (20), or 19 and 20,



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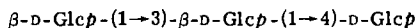


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and it was therefore proposed that the polysaccharide consists essentially of cellotriose units joined by (1→3)-β-D-glucosidic linkages in a linear fashion. The results of examination of oat glucan by similar techniques⁴⁰¹ suggested the same type of structure. A structure of this type for these glucans has been most convincingly confirmed by use of enzymic hydrolysis with^{45,47} cellulase and laminaranase. In addition, this work has provided additional information on the fine structures of these polysaccharides.

Degradation of oat and barley glucans with *Streptomyces* cellulase, which produces mainly cellobiose and cellotriose from cellulose,³⁵⁸ resulted in production of a trisaccharide fraction and a tetrasaccharide fraction as the major products.⁴⁵ These were isolated by preparative paper-chromatography, and the trisaccharide was found to preponderate;

it was present in an amount equivalent to 60% of the original polysaccharide, and the tetrasaccharide fraction accounted for 30%. The latter fraction was found to be a mixture of two oligosaccharides. Characterization of the products was achieved by formation of acetates, partial hydrolysis with acid, and an elegant lead tetraacetate oxidation procedure that showed them to be *O*- β -D-glucopyranosyl-(1 \rightarrow 3)-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)-D-glucopyranose (21), *O*- β -D-glucopyranosyl-(1 \rightarrow 4)-*O*- β -D-glucopyranosyl-(1 \rightarrow 3)-D-glucopyranose (22).



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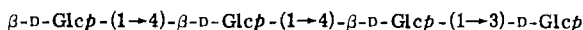
copyranosyl-(1 \rightarrow 3)-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)-D-glucopyranose (20), and *O*- β -D-glucopyranosyl-(1 \rightarrow 3)-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)-D-glucopyranose (19). Consideration of the structures of these three major products and their relative proportions, together with the linkage composition of the original polysaccharide, suggested a structure of the type shown diagrammatically in Fig. 20. As may be seen from Fig. 20a, it was not necessary to postulate the cleavage of any linkages other than (1 \rightarrow 4)- β -D-glucosidic linkages by the enzyme. The cereal polysaccharides are, therefore, essentially polymers of (mainly) cellotriose and, to a lesser extent, cellotetraose units, joined by (1 \rightarrow 3)- β -D-glucosidic linkages. No evidence was found for the presence of adjacent (1 \rightarrow 3)- β -D-glucosidic linkages, as indicated by the failure to detect laminaratriose among the hydrolysis products. A similar conclusion was reached on degradation of barley glucan by a purified (1 \rightarrow 4)- β -D-glucan hydrolase from germinated barley.^{371,402} Claims,^{403,404} based on the results of nonenzymic degradation, that contiguous (1 \rightarrow 3)- β -D-linkages exist in this polysaccharide are, therefore, open to question.

Degradation of the oat polysaccharide by a laminaranase [unspecific (1 \rightarrow 3)- β -D-glucanase] that produces mainly laminarabiose and laminaratriose from laminaran gave, as the major products, tri- and tetra-saccharide fractions,⁴⁵ the latter being homogeneous. The trisaccharide (50% of the glucan) was characterized as *O*- β -D-glucopyranosyl-(1 \rightarrow 4)-*O*- β -D-glucopyranosyl-(1 \rightarrow 3)-D-glucopyranose (17) by the same procedures used for the cellulase degradation products. The tetrasaccharide (30%) was shown to be *O*- β -D-glucopyranosyl-(1 \rightarrow 4)-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)-*O*- β -D-glucopyranosyl-(1 \rightarrow 3)-D-glucopyranose (22). To account for the

(402) J. J. Marshall, Ph. D. Thesis, Heriot-Watt University, Edinburgh, 1969.

(403) I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, *Abstr. Papers Amer. Chem. Soc. Meeting*, 135, 3D (1959).

(404) M. Fleming and D. J. Manners, *Biochem. J.*, 100, 4P (1966).



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high percentage of (1→3)-β-D-glucosidic linkages in the products, compared with that to be expected were (1→3)-β-D-glucosidic linkages split, it was postulated that the enzyme was specific for the cleavage of the linkage adjacent to a β-D-glucosyl residue substituted at O-3, rather than for cleavage of (1→3)-β-D-glucosidic linkages themselves, as might have been expected. The action of the enzyme on the polysaccharide is illustrated in Fig. 20b. No attempt was, however, made to prove the correctness of the hypothesis by testing the action of the enzyme on model oligosaccharides, but it was supported by the formation of only one

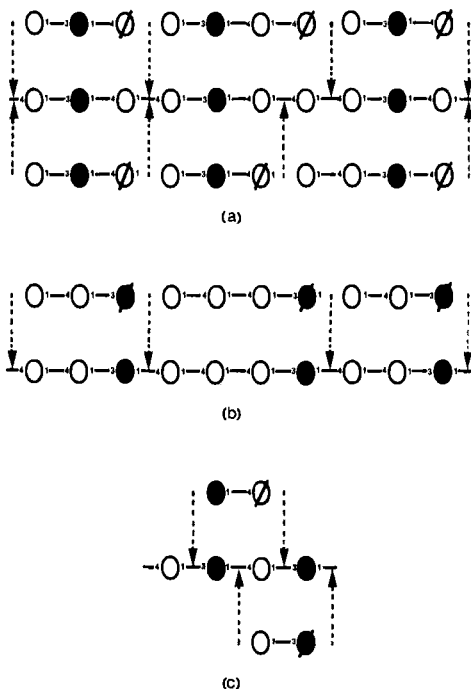


FIG. 20.—(a) The Degradation⁴⁵ of Mixed-linkage β-D-Glucans (Central Row of Circles) to Oligosaccharides by the Action of Cellulase; (b) Degradation of Mixed-linkage β-D-Glucans (Lower Row of Circles) by Laminaranase; (c) Production of Cellobiose and Laminarabiose from a Minor Structural Feature of Lichenan by the Action of Cellulase and Laminaranase. [The symbols are: ○, β-D-glucopyranose residue; and ∅, reducing β-D-glucopyranose residue. For contrast, those units bonded glycosidically through O-1 and O-3 are shown in black.]

tetrasaccharide. Although the presence of a mixture of polysaccharides, one containing only the tetrameric repeating-unit, and the other, only the pentameric unit, was not excluded, the formation of the two tetrasaccharides by cellulase was taken as indicating the presence of both structural features in the one molecule.

A subsequent study of the fine structure of lichenan⁴⁰⁵ by degradation with the same enzymes indicated some differences between this polysaccharide and the cereal glucans. This was apparent even before characterization of the products formed, just by determination of the relative proportions of the various oligosaccharides (dimer, trimer, and tetramer) produced (see Table IX). This result is not surprising in view of the higher ratio of (1→3)- to (1→4)- β -D-glucosidic linkages in lichenan as compared to cereal β -D-glucans.

The action of cellulase on lichenan produced cellobiose and a single trisaccharide, namely, O- β -D-glucopyranosyl-(1→3)-O- β -D-glucopyranosyl-(1→4)-D-glucopyranose (21). The latter was the preponderant oligosaccharide, produced in an amount accounting for 60% of the polymer. As with the cereal glucans, the tetrasaccharide fraction was heterogeneous. The components were characterized as O- β -D-glucopyranosyl-(1→4)-O- β -D-glucopyranosyl-(1→3)-O- β -D-glucopyranosyl-(1→4)-D-glucopyranose (20) and (as a minor constituent) O- β -D-glucopyranosyl-(1→3)-O- β -D-glucopyranosyl-(1→4)-O- β -D-glucopyranosyl-(1→4)-D-glucopyranose (19). *Rhizopus arrhizus* laminaranase produced, in an amount corresponding to 55% of the polysaccharide, the trisaccharide O- β -D-glucopyranosyl-(1→4)-O- β -D-glucopyranosyl-(1→3)-D-glucopyranose (17), together with laminarabiose, a tetrasaccharide, and higher oligosaccharides in minor proportions. Cunningham and Manners⁴⁰⁵ later detected the trisaccharide O- β -D-glucopyranosyl-(1→4)-O- β -D-glucopyranosyl-(1→3)-D-glucopyranose (17) as a degradation product of lichenan by an enzyme preparation from the same organism.

These findings clearly supported a structure of the type previously proposed for lichenan by Peat and coworkers,⁴⁰¹ consisting of a tetrameric repeating-unit containing two contiguous (1→4)- β -D-glucosidic linkages and a (1→3)- β -D-glucosidic linkage (see Fig. 20). The formation of the products of enzymic hydrolysis of a polysaccharide having this type of structure is also shown in Fig. 20; again, laminaranase action mainly, if not entirely, involves cleavage of (1→4)- β -D-glucosidic linkages. The formation of the two tetrasaccharides by action of cellulase also indicates the presence of sequences in which a single (1→3)- β -D-glu-

(405) W. L. Cunningham and D. J. Manners, *Biochem. J.*, **90**, 596 (1964).

TABLE IX
Products from the Enzymic Degradation of Lichenan and Cereal Glucans^a

<i>Enzyme</i>	<i>Substrate</i>	<i>Initial polymer (mg)</i>	<i>Dimer (mg)</i>	<i>Trimer (mg)</i>	<i>Tetramer (mg)</i>	<i>Trimer to tetramer</i>
(1→3)-β-D-Glucanase (laminaranase)	Lichenan	24.4	2.2	14.2	3.6	4.0
	Oat glucan	20.4	1.2	10.4	4.8	2.2
	Barley glucan	23.0	1.5	11.7	4.8	2.4
(1→4)-β-D-Glucanase (cellulase)	Lichenan	24.4	3.4	13.4	3.7	3.6 ^a
	Oat glucan	21.5	2.6	10.2	6.8	1.5 ^a
	Barley glucan	21.1	3.4	10.2	5.5	1.8 ^a

^a These are minimal values, as the tetramer fraction, particularly that from lichenan, contained a substantial proportion of material travelling on the chromatograms at a slightly lower rate.

cosidic linkage alternates with three consecutive (1→4)- β -D-linkages, although the latter structural feature is of less importance in lichenan than in the cereal glucans.

Evidence consistent with these conclusions came from a consideration of the ratios of trimer (from the former structural feature) to tetramer (from the latter structural feature) produced from the two types of polysaccharide (3.6:1 from lichenan and 1.7:1 from oat and barley glucans), as well as the periodate-oxidation data indicating the higher proportion of (1→3)- β -D-glucosidic linkages in lichenan. The conclusion was that, although the gross features of the cereal glucans and lichenan are similar, they may be differentiated in terms of fine structure. The production, from lichenan, of cellobiose (by cellulase action) and laminarabiose (by laminaranase action) in small proportions (in the order of 10%)⁴⁶ was accounted for by the suggestion that, in lichenan, an additional structural feature may be present, namely, a single (1→4)- β -D-glucosidic linkage between two (1→3)- β -D-glucosidic linkages (see Fig. 20c, p. 356). Comparison of lichenan with barley glucan by Smith degradation¹⁰² has also been claimed to be in agreement with the supposition that there are differences in the fine structures of these polysaccharides.⁴⁰⁴

Further enzymic evidence that barley glucan, oat glucan, and lichenan have structures of the type suggested by Peat and coworkers⁴⁰¹ and Perlman and coworkers^{45,47} came from degradation of these polysaccharides with a (1→4)- β -D-glucanase from *Aspergillus niger*^{356,357} that yielded cellobiose (12%), O- β -D-glucopyranosyl-(1→3)-O- β -D-glucopyranosyl-(1→4)-D-glucopyranose (21) (45%), and a tetrasaccharide fraction that was a substituted cellobiose (16%).⁴⁰⁶ Degradation of lichenan (10% hydrolysis) was less than that of the cereal β -D-glucans (14–15% hydrolysis), alcohol-precipitable material remaining in the enzyme digest,⁴⁰⁶ confirming the difference in the fine structures of these polysaccharides. Similar results obtained by using cellulase from *Trichoderma viride* have been reported,⁴⁰⁷ but the production of over 20% of D-glucose from barley glucan made the significance of the work questionable, as it probably indicated the presence of contaminating β -D-glucosidase in the enzyme preparation used.

Degradation of barley glucan by an enzyme (a laminaranase similar to that from *Rhizopus arrhizus*) from *Bacillus circulans*³⁷⁸ yielded O- β -D-

(406) A. E. Clarke and B. A. Stone, *Biochem. J.*, **99**, 582 (1966).

(407) O. Igarashi, M. Noguchi, and M. Fujimaki, *Agr. Biol. Chem. (Tokyo)*, **32**, 272 (1968).

glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→3)-D-glucopyranose (17) (59%) and O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→3)-D-glucopyranose³⁷⁹ (22). Degradation by a purified, barley enzyme, claimed to be specific for degradation of mixed-linkage [(1→3):(1→4)]-β-D-glucans [and, therefore, similar to the bacterial enzyme (lichenase)³⁸⁹ used by Moscatelli and co-workers³⁸⁸ for degradation of barley glucan, and by Reese and Perlin³⁸⁷ for degradation of lichenan], gave the same products as the *Bacillus circulans* enzyme, in similar yields.^{408,409} These reports confirmed the existence in barley glucan of two types of repeating unit, trimeric and tetrameric. In neither of these studies was there any evidence for production of laminaratriose, thus confirming the absence of contiguous (1→3)-β-D-glucosidic linkages in these glucans.

It remains to be ascertained whether there are any significant differences between the fine structures of oat and barley glucans. Preece⁴¹⁰ has claimed that there are, single (1→3)-β-D-glucosidic linkages in the former being absent, or few in number; this view is in direct contrast to Perlin's.⁴⁷ The answer to the question must, therefore, await further investigations. It seems to the writer that a simple way to look for fine-structure differences between these various, mixed-linkage glucans would be to compare the products of enzymic degradation of each by using a "fingerprinting" technique. This could be conveniently done (after degradation of the different glucans by the various enzymes) by molecular-sieve chromatography on columns of a suitable Biogel or Sephadex, followed by a comparison of the elution profiles for each (compare p. 322). In this way, any differences in fine structure might become apparent.

The use of β-D-glucan hydrolases in the characterization of plant β-D-glucans synthesized *in vitro* is demonstrated by the characterization of an alkali-insoluble glucan, produced from UDP-D-glucose by a particulate enzyme preparation from *Phaseolus aureus*,⁴¹¹ as of the callose type, by using Basidiomycete *exo*-(1→3)-β-D-glucanase. Despite the insolubility of the polysaccharide, it was hydrolyzed to the extent of 91% in 24 hr, confirming that it is a (1→3)-β-D-glucan.⁴¹¹ In contrast, the alkali-insoluble glucan formed from GDP-D-glucose by the same enzyme preparation was not degraded.⁴¹¹ These findings therefore supported earlier,

(408) W. W. Luchsinger, S.-C. Chen, and A. W. Richards, *Arch. Biochem. Biophys.*, **112**, 524 (1965).

(409) W. W. Luchsinger, S.-C. Chen, and A. W. Richards, *Arch. Biochem. Biophys.*, **112**, 531 (1965).

(410) I. A. Preece and N. K. Garg, *J. Inst. Brewing*, **67**, 267 (1961).

(411) K. K. Batra and W. Z. Hassid, *Plant Physiol.*, **44**, 755 (1969).

nonenzymic studies that had suggested that the latter polysaccharide contains (1→4)- β -D-glucosidic linkages; that is, that it is a cellulose-type polysaccharide.^{412,413} In a similar way, the glucan synthesized *in vitro* from UDP-D-glucose by a preparation from *Lupinus albus* was degraded to near completion by the Basidiomycete enzyme, and was, therefore, also (1→3)- β -D-linked.⁴¹⁴ In contrast, that produced from the same "sugar nucleotide" by an enzyme preparation from *Avena sativa* was only partly (70%) degraded in the same time. Although this result was considered by the authors not to be inconsistent with previous suggestions^{415,416} that the latter polysaccharide contains both (1→3)- and (1→4)- β -D-glucosidic linkages, the possibility that these linkages were present in different polysaccharides could not be excluded. The alkali-insoluble nature of the polysaccharide would, however, suggest heterogeneity, as mixed-linkage glucans are water-soluble. Furthermore, degradation of a mixed-linkage polysaccharide to the extent of 70% by the Basidiomycete enzyme would be possible only if the linkages were so arranged that the (1→3)- β -D-links preponderated at the nonreducing end and the (1→4)- β -D-links at the reducing end of the polysaccharide, because this enzyme does not act on oat and barley glucans.³⁶ The problem could be readily solved by use of other glucanases, such as those already mentioned, or by examination of the product left after *exo*-(1→3)- β -D-glucanase action has ceased.

b. Microbial Cell-wall Polysaccharides.—The nature of the glucan component of the cell wall of the yeast *Saccharomyces cerevisiae* has attracted considerable attention for a number of years. Although its nature as essentially a (1→3)- β -D-glucan has long been realized,^{417,418} the elucidation of its fine structure did not prove possible by nonenzymic methods, but was achieved by the application of appropriate enzymes.

Early claims⁴¹⁹ that the glucan contains (1→2)- β -D-glucosidic linkages, in addition to the (1→3)- β -D-glucosidic linkages, were not substantiated by later work⁴²⁰ that suggested that the other D-glucosidic linkages present are, in fact, (1→6)- β -D in nature. Partial hydrolysis of the glucan⁴²⁰ with

(412) A. D. Elbein, G. A. Barber, and W. Z. Hassid, *J. Amer. Chem. Soc.*, **86**, 309 (1964).

(413) G. A. Barber, A. D. Elbein, and W. Z. Hassid, *J. Biol. Chem.*, **239**, 4056 (1964).

(414) K. K. Batra and W. Z. Hassid, *Plant. Physiol.*, **45**, 233 (1970).

(415) L. Ordin and M. A. Hall, *Plant Physiol.*, **42**, 205 (1967).

(416) L. Ordin and M. A. Hall, *Plant Physiol.*, **43**, 473 (1968).

(417) L. Zechmeister and G. Tóth, *Biochem. Z.*, **270**, 309 (1934).

(418) L. Zechmeister and G. Tóth, *Biochem. Z.*, **284**, 133 (1936).

(419) D. J. Bell and D. H. Northcote, *J. Chem. Soc.*, 1944 (1950).

(420) S. Peat, W. J. Whelan, and T. E. Edwards, *J. Chem. Soc.*, 3862 (1958).

acid gave products [laminara-oligosaccharides, gentio-oligosaccharides, and some oligosaccharides containing both (1→3)- β -D- and (1→6)- β -D-glucosidic linkages] which indicated that the glucan was linear, there being no evidence for any oligosaccharides arising from branch points. The earlier methylation results,⁴¹⁹ which had indicated a highly branched structure, the branch points being (1→2)- β -D-glucosidic linkages, were attributed to undermethylation.

Treatment of yeast glucan with an extremely crude, bacterial laminar-anase preparation from a *Cytophaga* species, which, although containing a mixture of several α - and β -D-glucan hydrolases,³⁷⁴ had no action on (1→6)- β -D-glucans, left a macromolecular limit-dextrin.⁴²¹ This dextrin was characterized as a (1→6)- β -D-glucan by methylation analysis, partial hydrolysis with acid, and enzymic hydrolysis with a (1→6)- β -D-glucanase preparation from *Penicillium brefeldianum*^{389a} which gave a series of gentio-oligosaccharides. It was concluded, on the basis of this result and a not-very-convincing, methylation analysis of the native glucan, that the polysaccharide was highly branched, with a (1→6)- β -D-linked backbone and (1→3)- β -D-linked side-chains.

A subsequent study of the yeast glucan and its periodate-oxidized derivative, by Smith and coworkers,⁴²² using periodate oxidation, methylation analysis, and enzymic hydrolysis with *Rhizopus arrhizus* (1→3)- β -D-glucanase, was also taken to indicate a branched structure. These workers were, however, unable to detect any significant proportion of sequences of (1→6)-linked β -D-glucose residues, and suggested that the glucan was essentially (1→3)- β -D in nature, with (1→6)- β -D inter-chain linkages.

The problem was resolved, and the inconsistencies explained, by Bacon and coworkers, who showed^{423,424} that a (1→6)- β -D-glucan component could be removed from alkali-extracted yeast-glucan by treatment with a commercial, chitinase preparation contaminated with (1→6)- β -D-glucanase, suggesting the presence, in yeast-glucan preparations, of a polysaccharide distinct from the (1→3)- β -D-glucan. It was further shown that the (1→6)- β -D-glucan component could be removed from alkali-extracted yeast-glucan by extraction of the latter with dilute acetic acid. As Manners and Patterson⁴²¹ had used the conventional, alkaline-extraction method⁴¹⁹ for preparation of their glucan, and Smith and coworkers

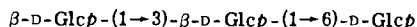
(421) D. J. Manners and J. C. Patterson, *Biochem. J.*, **98**, 19c (1966).

(422) A. Misaki, J. Johnson, S. Kirkwood, J. V. Scaletti, and F. Smith, *Carbohydr. Res.*, **6**, 150 (1968).

(423) J. S. D. Bacon and V. C. Farmer, *Biochem. J.*, **110**, 34p (1968).

(424) J. S. D. Bacon, V. C. Farmer, D. Jones, and I. F. Taylor, *Biochem. J.*, **114**, 557 (1969).

had prepared theirs by a procedure involving much more extensive extraction with acetic acid, it became apparent that these two groups of workers were dealing with yeast-glucan preparations containing different proportions of the contaminating (1→6)- β -D-glucan. Thus, it became clear that (1→6)- β -D-linked D-glucose residues do not constitute a significant proportion of the linkages in yeast glucan, and certainly not its backbone, as Manners and Patterson had suggested.⁴²¹ Subsequent work⁴²⁵ has confirmed that the material extracted with acetic acid is essentially a linear (1→6)- β -D-glucan having a small number of (1→3)-linked β -D-glucose residues. Thus, the glucan is not degraded by a laminaranase preparation, but *Penicillium brefeldianum* (1→6)- β -D-glucanase^{389a} degrades it to D-glucose, gentio-oligosaccharides, and the trisaccharide O- β -D-glucopyranosyl-(1→3)-O- β -D-glucopyranosyl-(1→6)-D-glucopyranose (23). On periodate oxidation, the glucan consumes 2.1



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molecular proportions of periodate per D-glucose residue. Smith degradation yields, in addition to glycerol, a D-glucosylglycerol arising from the D-glucose residues substituted at O-3.

After removal, with *Penicillium brefeldianum* (1→6)- β -D-glucanase, of traces of the (1→6)- β -D-glucan remaining in the alkali-insoluble material after extraction with acetic acid, the residue was characterized as essentially (1→3)- β -D in nature by partial hydrolysis (acidic and enzymic); this gave a mixture of laminara-oligosaccharides, together with D-glucose and a small proportion of gentiobiose. It was claimed that the molecule was slightly branched, although the results did not clearly distinguish between (1→6)- β -D-glucosidic linkages present as branch points or (as originally suggested by Peat and coworkers⁴⁰¹) in a linear chain.

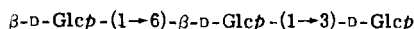
On degradation, supposedly with Basidiomycete *exo*-(1→3)- β -D-glucanase containing D-glucono-1,5-lactone to inhibit β -D-glucosidase activity present as an impurity, an alkali-soluble glucan from the cell walls of *Pullularia pullulans* gave, as products, D-glucose, laminarabiose, and two higher oligosaccharides, at least one of which contained a (1→6)- β -D-glucosidic linkage.⁴²⁶ After Smith degradation, enzymic hydrolysis with the same enzyme gave only D-glucose and laminarabiose. These findings, together with those from methylation analysis, were taken to indicate

(425) D. J. Manners and A. J. Masson, *FEBS Lett.*, **4**, 122 (1969).

(426) R. G. Brown and B. Lindberg, *Acta Chem. Scand.*, **21**, 2379 (1967).

that the structure of the polysaccharide consists of a (1→3)-linked β -D-glucan backbone with (1→6)- β -D-glucosyl side-chains at intervals of, on average, about every ninth D-glucose residue in the backbone. It is, however, difficult to account for the production of laminarabiose and the other oligosaccharides by the action of this *exo*-enzyme. No attempt was made to explain the fact that the production of these oligosaccharides was not in accord with the known specificity of the enzyme (see p. 351). Assuming the enzyme used was, in fact, an *exo*-(1→3)- β -D-glucanase (no control digests with substrates of known structure were conducted), these results cannot be considered to be in agreement with a structure of the type proposed, as this would have yielded only D-glucose and gentiobiose (compare Ref. 381).

Partial characterization of a cell-wall polysaccharide from *Piricularia oryzae* was achieved by Nakajima and coworkers⁴²⁷ with the aid of an enzyme preparation from *Bacillus circulans* that was known to contain *endo*-(1→3)- β -D-glucanase and (1→6)- β -D-glucanase, and also separately with Basidiomycete *exo*-(1→3)- β -D-glucanase. The former enzyme preparation produced D-glucose and laminarabiose; the latter gave the same sugars, together with O- β -D-glucopyranosyl-(1→6)-O- β -D-glucopyranosyl-(1→3)-D-glucopyranose (24). Together with the results of



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acetolysis, which led to D-glucose, laminarabiose, and laminaratriose, it was concluded that the glucan was (1→3)- β -D in nature, with (1→6)- β -D branch points. These workers, in contrast to Brown and Lindberg,⁴²⁶ realized the difficulty of accounting for the oligosaccharides produced by the *exo*-(1→3)- β -D-glucanase. Control experiments with the (1→3)- β -D-glucan pachyman³⁷⁵ and with schizophylan, a (1→3)- β -D-glucan having (1→6)- β -D-glucosyl side-chains (see p. 368), gave the expected products, namely, D-glucose for the first glucan, and D-glucose plus gentiobiose for the second. It was concluded that the *Piricularia* cell-wall glucan⁴²⁷ has a structure more complicated than that of schizophylan, and it was suggested that (1→3)- β -D-glucosidic linkages resistant to the action of *exo*- β -D-glucanase were present. However, there was insufficient evidence to permit proposing a structure for the polysaccharide.

It is important that the action of this *exo*-enzyme on (1→3:1→6)- β -D-linked polysaccharides, which are an important type of fungal glucan,

(427) T. Nakajima, K. Tamari, K. Matsuda, H. Tanaka, and N. Ogasawara, *Agr. Biol. Chem.* (Tokyo), **34**, 553 (1970).

be clarified. Examination of the action of the enzyme on a range of model oligosaccharides wider than has been studied previously may be enlightening. Clearly, however, the polysaccharides from *Pullularia pullulans* and *Piricularia oryzae* cell-walls are, on the basis of the results of degradation with this enzyme, similar in structure, and not merely (1→3)- β -D-glucans having single, (1→6)-linked, β -D-glucosyl side-chains. It is not possible to reach any firmer conclusions until the detailed specificity of the enzyme has been established.

Action of a crude, β -D-glucan hydrolase preparation from *Streptomyces*⁴²⁸ on the hyphal walls of the fungus *Phytophthora cinnamomi* produced, among other compounds, cellobiose, laminarabiose, and gentiobiose.⁴²⁸ This result was considered to suggest the presence of cellulose, together with a glucan containing (1→3)- and (1→6)- β -D-glucosidic linkages. The latter polysaccharide was isolated, and analysis⁴²⁹ showed it to be a highly branched polysaccharide containing 15–20% of nonreducing end-groups, 45–50% of D-glucose residues substituted at O-3, 10% of D-glucose residues substituted at O-4, and 20% of D-glucose residues substituted at both O-3 and O-6. Degradation of this insoluble polysaccharide⁴²⁹ with Basidiomycete *exo*-(1→3)- β -D-glucanase resulted in almost complete solubilization, and liberation, as the major products, of D-glucose (33%) and an alcohol-precipitable limit-dextrin (20%). The limit dextrin, which contained approximately equal proportions of (1→3)- and (1→6)- β -D-glucosidic linkages, was not further characterized. These findings, together with nonenzymic analyses, suggested that the glucan was branched with side chains of (1→3)-linked β -D-glucose residues (~4–5 per chain) linked to a backbone containing (1→3)- or (1→6)- β -D-glucosidic linkages, or both. A structure similar to that proposed for yeast glucan by Smith and coworkers⁴²² was suggested for the polysaccharide, but it was not possible to decide whether the proportion of (1→4)- β -D-glucosidic linkages present constitute part of the *Phytophthora* glucan, or merely indicate the presence of contaminating cellulose.

Enzymic hydrolysis of glucans, considered to contain (1→6)- β -D-glucosidic linkages, from other oomycetous fungi also yielded D-glucose.⁴³⁰ However, as the enzyme preparation [described, paradoxically, as a (1→3)- β -D-glucanohydrolase which acted in an *exo* fashion, releasing D-glucose] was not identified, and the extents of degradation were not indicated, these results are of little significance. Indirect, and rather unsatisfactory, evidence for the presence of two components in the cell wall of the oomycetous fungus *Pythium* was obtained by separate treatment

(428) S. Bartnicki-Garcia and E. Lippman, *Biochim. Biophys. Acta*, **136**, 533 (1967).

(429) L. P. T. M. Zevenhuizen and S. Bartnicki-Garcia, *Biochemistry*, **8**, 1496 (1969).

(430) J. M. Aronson, B. A. Cooper, and M. S. Fuller, *Science*, **155**, 332 (1967).

of whole cells with two types of β -D-glucanase preparation.⁴³¹ *Streptomyces endo*-(1 \rightarrow 3)- β -D-glucanase yielded (1 \rightarrow 3)- β -D-linked oligosaccharides, and this result was taken to indicate the presence of a (1 \rightarrow 3)- β -D-glucan. Basidiomycete *exo*-(1 \rightarrow 3)- β -D-glucanase gave D-glucose and gentiobiose, suggesting the presence of a (1 \rightarrow 3:1 \rightarrow 6)- β -D-glucan of the type mentioned previously, that is, a glucan consisting of a (1 \rightarrow 3)- β -D-linked backbone with side chains of single, (1 \rightarrow 6)- β -D-linked, D-glucose residues. Although these results are not, in themselves, satisfactory proof of the existence of two glucans, they at least serve as a reminder that this possibility must be considered, particularly after the experiences with yeast glucan (see p. 362); this possibility was not considered for the *Phytophthora cinnamomi* cell-wall glucan.⁴²⁹

There are many other examples (see, for instance, Refs. 432–434) of the use of both *exo*- and *endo*-(1 \rightarrow 3)- β -D-glucanases to show the presence of (1 \rightarrow 3)- β -D-glucans in fungal cell-walls. Seldom, however, have the polysaccharides been extracted and purified; instead, the enzymes have usually been used on the complete cell-wall. For example, in an effort to characterize fungal, cell-wall polysaccharides by methods involving treatment much more gentle than that used previously, both for extraction and analysis, cell walls prepared from *Penicillium chrysogenum* by glycerol shock-treatment followed by disintegration by use of glass beads were analyzed enzymically.⁴³⁵ Degradation of the cell-wall polysaccharide with a (1 \rightarrow 3)- β -D-glucanase preparation from *Bacillus circulans* released only oligosaccharides containing (1 \rightarrow 3)- β -D-glucosidic linkages. The absence of oligosaccharides containing both (1 \rightarrow 3)- and (1 \rightarrow 4)- β -D-glucosidic linkages showed the absence of any polymer of the lichenan type. From the degree of solubilization of the cell wall by the (1 \rightarrow 3)- β -D-glucanase, it was estimated that (1 \rightarrow 3)- β -D-glucan accounted for 40% of the cell wall. When the constituent glucans of this and other fungi have been obtained in a purified state, it is likely that the same enzymes, in conjunction with nonenzymic examinations, will prove of great value in determination of their fine structures.

c. Extracellular and Reserve β -D-Glucans.—Several micro-organisms have been shown to produce (1 \rightarrow 3:1 \rightarrow 6)- β -D-glucans having closely related structures. One of the first polysaccharides of this type (sclerotan) was isolated from the sclerotia of *Sclerotinia libertiana* by Japanese

(431) D. E. Eveleigh, J. H. Sietsma, and R. H. Haskins, *J. Gen. Microbiol.*, **52**, 89 (1968).

(432) H. J. Potgieter and M. Alexander, *Can. J. Microbiol.*, **11**, 122 (1965).

(433) J. J. Skujins, H. J. Potgieter, and M. Alexander, *Arch. Biochem. Biophys.*, **111**, 358 (1965).

(434) P. R. Mahadevan and U. R. Mahadkar, *Indian J. Exp. Bot.*, **8**, 207 (1970).

(435) F. A. Troy and H. Koffler, *J. Biol. Chem.*, **244**, 5563 (1969).

workers.^{436,436a} On the basis of nonenzymic studies, it was assigned a structure consisting of a (1→3)- β -D-linked backbone with single (1→6)- β -D-linked side-chains. Subsequent enzymic-degradation studies,^{386,436b} with an *exo*-(1→3)- β -D-glucan hydrolase from cultures of the same organism, showed that the enzyme yielded D-glucose and gentiobiose, lending support to the structure proposed.

Smith and coworkers⁴³⁷ used enzymic degradation to investigate the structure of a closely similar polysaccharide produced by *Sclerotium rolfsii*^{437a} (see Fig. 21). Treatment with Basidiomycete *exo*-(1→3)- β -D-glucanase brought about at least 90% conversion into D-glucose and gentiobiose, formed in the ratio of 2:1; *Rhizopus arrhizus* (1→3)- β -D-glucanase gave D-glucose, gentiobiose, and laminarabiose, but no laminaratriose. Smith degradation of the glucan yielded an insoluble product that, by action of these two enzymes, yielded, respectively, D-glucose alone, and D-glucose, laminarabiose, and laminaratriose. On the basis of these enzymic-degradation studies, with support from the results of methylation analysis, it was proposed that the polysaccharide is a (1→3)- β -D-glucan having single, (1→6)- β -D-glucosyl side-chains distributed evenly along the chain on every third D-glucose residue.

On degradation with Basidiomycete *exo*-(1→3)- β -D-glucanase, the extracellular polysaccharide from *Claviceps purpurea*⁴³⁸ gave D-glucose and gentiobiose in the molar ratio of 3:1, but the polysaccharide was resistant to degradation by *Rhizopus arrhizus endo*-(1→3)- β -D-glucanase. A highly insoluble glucan was obtained by removal of the oxidizable D-glucose residues by Smith degradation; this was a (1→3)- β -D-glucan. The oxidizable units were, therefore, present as end groups. It was concluded that these were present as single, (1→6)-linked β -D-glucosyl groups on, approximately, every fourth unit in the (1→3)-backbone. By use of the Basidiomycete enzyme, together with nonenzymic analyses, the polysaccharide produced extracellularly by *Pullularia pullulans* growing on D-xylose was characterized^{438a} as a β -D-glucan of the

(436) M. Kitahara and Y. Takeuchi, *Gifu Daigaku Nogakubu Kenkyu Hokoku*, **11**, 127 (1959); *Chem. Abstr.*, **54**, 16,529d (1960).

(436a) M. Kitahara and Y. Takeuchi, *Nippon Nogei Kagaku Kaishi*, **35**, 474 (1961).

(436b) Y. Satomura, M. Ono, and J. Fukumoto, *Bull. Agr. Chem. Soc. Japan*, **24**, 317 (1960).

(437) J. Johnson, S. Kirkwood, A. Misaki, T. E. Nelson, J. V. Scaletti, and F. Smith, *Chem. Ind.* (London), 820 (1963).

(437a) Although the source of the polysaccharide was not given in the original article (Ref. 437), it was subsequently identified as *Sclerotium rolfsii*, and the polysaccharide was referred to as sclerotan.³⁸¹

(438) A. S. Perlin and W. A. Taber, *Can. J. Chem.*, **41**, 2278 (1963).

(438a) H. O. Bouveng, H. Kiessling, B. Lindberg, and J. McKay, *Acta Chem. Scand.*, **17**, 1351 (1963).

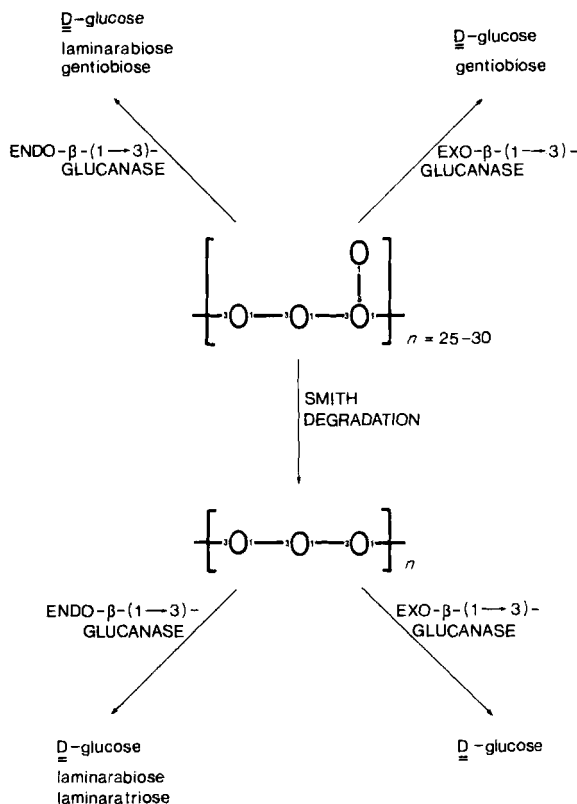


FIG. 21.—Enzymic Degradation of *Sclerotium rolfii* β -Glucan Before and After Smith Degradation. [The symbols are as given for Fig. 20.]

same type, but having two out of three D-glucose residues in the backbone carrying substituents. The extent of enzymic hydrolysis of this polysaccharide was relatively low (26–38%), in agreement with the high degree of substitution of the residues in the main chain. *Claviceps fusiformis* produces a similar β -D-glucan,⁴³⁹ and a polysaccharide (schizophyllan) of the same general type is formed by *Schizophyllum commune*.^{440,441} The latter glucan was degraded by both *endo*-(1 \rightarrow 3)- β -D-glucanase from *Bacillus circulans* (42% hydrolysis) and by *exo*-(1 \rightarrow 3)-

(439) K. W. Buck, A. W. Chen, A. G. Dickerson, and E. B. Chain, *J. Gen. Microbiol.*, **51**, 337 (1968).

(440) S. Kikumoto, T. Miyajima, S. Yoshizumi, S. Fujimoto, and K. Kimura, *Nippon Nogei Kagaku Kaishi*, **44**, 337 (1970).

(441) S. Kikumoto, T. Miyajima, K. Kimura, S. Okubo, and N. Komatsu, *Nippon Nogei Kagaku Kaishi*, **45**, 162 (1971).

β -D-glucanase from Basidiomycete (63% hydrolysis), giving the expected products in both cases.^{441,442}

A soluble, cytoplasmic glucan from *Phytophthora cinnamomi*, an important reserve material of the fungus, gave, on degradation with a crude, glucanase mixture from *Streptomyces*, a series of laminara-oligosaccharides of d.p. up to 6, as well as higher oligosaccharides.⁴⁴³ No values for the extent of degradation were, however, given. From these results, together with those from nonenzymic studies, it was concluded that the predominant, structural feature of this glucan is the presence of (1 \rightarrow 3)- β -D chains with (1 \rightarrow 6)- β -D branch-points, there being approximately eight (1 \rightarrow 3)-linked β -D-glucose residues per nonreducing end-group. However, the fine structure of the polysaccharide has not yet been determined. The high solubility of the glucan in water suggests that this polysaccharide may consist of a large number of relatively short chains, interlinked by the (1 \rightarrow 6)- β -D-glucosidic linkages; that is, it is analogous to glycogen, rather than having a D-glucosyl-substituted backbone.

In attempts to compare the fine structures of the (1 \rightarrow 3)- β -D-glucans curdlan and pachyman,⁴⁴⁴ and to account for their somewhat different gelation properties, the products of degradation of each by *Rhizopus arrhizus* and Basidiomycete glucanases were examined. No differences were apparent. When the sizes of these polysaccharides are considered (degrees of polymerization in the range of 250–450) in conjunction with the fact that the differences turned out to be attributable to the minute proportion of (1 \rightarrow 6)- β -D-glucosidic linkages (present mainly as branch points in the pachyman molecules, but as linkages in a linear chain in curdlan), it is hardly surprising that these minor, structural features were not identified enzymically. Characterization of these minor features in this way would be as difficult as characterization of the nature of the (1 \rightarrow 6)- α -D-glucosidic linkages in amylose (see p. 307) by examination of *alpha*-amylase limit-dextrins from the latter polysaccharide. An *exo*-(1 \rightarrow 3)- β -D-glucanase whose action is halted by (1 \rightarrow 6)- β -D-glucosidic linkages would be useful in this work, but no enzyme having this specificity is known.

Isosclerotan, an alkali-soluble polysaccharide from the sclerotia of *Sclerotinia libertiana*, was found⁴⁴⁵ to contain (1 \rightarrow 3)-, (1 \rightarrow 4)-, and

(442) T. Miyajima, S. Yoshizumi, S. Kikumoto, and H. Takahashi, *Seito Gijutsu Kenkyukaishi*, **22**, 35 (1970).

(443) L. P. T. M. Zevenhuizen and S. Bartnicki-Garcia, *J. Gen. Microbiol.*, **61**, 183 (1970).

(444) H. Saito, A. Misaki, and T. Harada, *Agr. Biol. Chem. (Tokyo)*, **32**, 1261 (1968).

(445) S. Oi, I. Konishi, and Y. Satomura, *Agr. Biol. Chem. (Tokyo)*, **30**, 266 (1966).

(1→6)- β -D-glucosidic linkages in the approximate ratios of 48:17:36. Nonenzymic analysis also showed the branched nature of the polysaccharide. There was little (8%) degradation of the polysaccharide by an *endo*-(1→3)- β -D-glucanase from *Fusarium*, but *Sclerotium libertiana* *exo*-(1→3)- β -D-glucanase brought about 35% hydrolysis (measured as D-glucose), although one of the major products appeared to be laminararibiose.⁴⁴⁵ Cellulase caused 13% hydrolysis. It was claimed that these results, together with those from nonenzymic studies, indicated the presence of a main chain of (1→3)- and (1→4)- β -D-linked D-glucose residues, with (1→6)- β -D branch-points; the evidence was not, however, convincing. *exo*-(1→3)- β -D-Glucanase would not be expected to degrade such a polysaccharide to any significant extent, unless the enzyme has a specificity different from that of the Basidiomycete enzyme.³⁶ This work illustrates the uncertainties that may arise from the use of inadequately characterized enzymes.

During routine studies that have characterized reserve, microbial polysaccharides as being of the paramylon type [that is, linear (1→3)- β -D-glucans], (1→3)- β -D-glucanases, particularly that from *Rhizopus arrhizus*,^{78,446} have frequently been used.

Studies on laminaran by enzymic methods have not yielded many meaningful results. One attempt⁴⁴⁷ must be disregarded in view of the demonstration that the enzyme preparation used contained a multiplicity of β -D-glucan hydrolases having different specificities.³⁷⁴ Use of suitable enzymes may prove a convenient method of examining in greater detail the differences in structure^{448,449} between the soluble and insoluble forms of laminaran.

(446) A. R. Archibald, W. L. Cunningham, D. J. Manners, J. R. Stark, and J. F. Ryley, *Biochem. J.*, **88**, 44 (1963).

(447) M. Fleming, D. J. Manners, and A. Masson, *Biochem. J.*, **104**, 32P (1966).

(448) W. D. Annan, Sir Edmund Hirst, and D. J. Manners, *J. Chem. Soc.*, 885 (1965).

(449) M. Fleming and D. J. Manners, *Biochem. J.*, **94**, 17P (1965).

DEXTRANS*

BY RAMON L. SIDEBOTHAM

*Department of Chemistry, Royal Holloway College, London University,
Englefield Green, Surrey TW20 0EX, England***

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I. INTRODUCTION

In this article, the term dextran will be used to describe preparations of D-glucans that contain substantial numbers of (1→6)-linked α -D-glucopyranosyl residues. In order to attain an element of cohesion between its various Sections, the Chapter has been confined to dextrans produced by bacteria growing on a sucrose substrate. The great majority of dextrans fall into this group, although dextrans have been synthesized from other substrates^{1,2} by micro-organisms, and a chemical synthesis of an essentially unbranched dextran has been reported.^{3,4}

Chapters in two Volumes of this Series,^{5,6} and in other series,^{7,8} dis-

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** Present address: *Department of Chemistry, Queen Elizabeth College, London University, Campden Hill Road, London W8 7AH, England.*

(1) S. A. Barker, E. J. Bourne, G. T. Bruce, and M. Stacey, *J. Chem. Soc.*, 4414 (1958).

(2) E. J. Hehre and D. Hamilton, *Proc. Soc. Exp. Biol. Med.*, **71**, 336 (1949).

(3) E. R. Ruckel and C. Schuerch, *J. Org. Chem.*, **31**, 2233 (1966).

(4) E. R. Ruckel and C. Schuerch, *Biopolymers*, **5**, 515 (1967).

(5) T. H. Evans and H. Hibbert, *Advan. Carbohydr. Chem.*, **2**, 203 (1946).

cussed the general literature up to 1960. The present article will, therefore, be primarily concerned with developments since that date, but necessarily includes some earlier material. The format adopted is similar to that used in Volume 15 of this Series,⁹ although some additional Sections of particular interest have been included.

Dextrans are commercially important polysaccharides,⁹ and there is now a considerable volume of literature devoted to the industrial production and numerous uses of native dextrans, partially degraded dextrans, and their derivatives, but these aspects have not been included in the present Chapter as they have formed the basis of several specialist articles^{10a-18b} since 1959.

II. ORIGIN AND PREPARATION

Large numbers of bacteria synthesize dextrans exocellularly when grown on sucrose-containing media. These bacteria have been confined

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- (6) W. B. Neely, *Advan. Carbohydr. Chem.*, **15**, 341 (1960).
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to the family Lactobacillaceae¹⁹ and, more specifically, to the genera *Lactobacillus*, *Leuconostoc*, and *Streptococcus*. Species of these genera that are reported to elaborate dextrans are listed in Table I.

Jeanes and her associates^{21,41-44} have outlined conditions suitable for

TABLE I
Species of Bacteria that Synthesize Dextrans

<i>Lactobacillus</i>	<i>Leuconostoc</i>	<i>Streptococcus</i>
<i>L. acidophilus</i> ²⁰	<i>L. dextransicum</i> ²¹⁻²³	<i>S. bovis</i> ²⁴⁻²⁶
<i>L. brevis</i> ^{27,28}	<i>L. mesenteroides</i> ²¹⁻²³	<i>S. chollis</i> ²⁹
<i>L. casei</i> ³⁰		<i>S. faecalis</i> ³¹
<i>L. musicus</i> ³²		<i>S. mitis</i> ³³
<i>L. pastorianus</i> ³⁴		<i>S. mutans</i> ^{20,35-37}
<i>L. viridescens</i> ³⁸		<i>S. sanguis</i> ^{26,37}
		<i>S. viridans</i> var. ^{21,39,40}

- (19) R. S. Breed, E. G. D. Murrey, and N. R. Smith, "Bergey's Manual of Determinative Bacteriology," Williams and Wilkins Co., Baltimore, Md., 7th Edition, 1957, p. 505.
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the production of *Leuconostoc* dextrans from bacterial cultures and cell-free enzyme extracts, and have developed procedures for isolating the polysaccharides from fermentation mixtures. Somewhat different conditions are required for the optimal production of streptococcal dextrans,^{35,45-49} and several schemes for extracting these dextrans from fermentation mixtures have been reported.^{35,37,45,47}

Purified *Leuconostoc* dextrans are usually prepared by deproteinizing the polysaccharides isolated from fermentation mixtures, and fractionally precipitating them several times from aqueous alcohols or ketones.^{21,41,42,44,50,51}

Particular difficulties exist, however, in purifying dextrans synthesized by many streptococci, because the bacteria simultaneously elaborate exocellular fructans (levans).^{35,37,52,53} Dextrans and levans synthesized concomitantly by *Leuconostoc* micro-organisms have been separated by fractionally precipitating the polysaccharides from aqueous ethanol.^{42,54} These separative procedures are, however, only effective when considerable differences in molecular weight exist between the main fractions of the two polysaccharides, and, as a result, they are of limited value in effecting separation of levans from many streptococcal dextrans. Reports that some oral bacteria are able to metabolize levans^{52,55-59} suggest that

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the removal of levans from streptococcal dextran preparations may be facilitated by treating the preparations with bacterial levanases.⁵⁹

Under the conditions required for dextran synthesis, many streptococci synthesize large amounts of endocellular, glycogen-like polysaccharides^{35,55} that may become significant contaminants of dextrans from whole-cultures of the bacteria³⁶ if sufficient bacterial cells lyse. Isolation of the dextrans by mild, extractive procedures^{35,37,47} before the bacteria enter the decline phase of their growth⁶⁰ should minimize contamination from this source, and also lessen the possibility that the dextrans will be degraded by endocellular enzymes.⁶¹ Mixtures of dextrans and glycogens have also been resolved by fractionally precipitating the polysaccharides from alkaline solutions with cetyltrimethylammonium bromide (Cetavlon),⁶² and as their copper complexes.⁶³

It should be emphasized that the separative procedures that have been outlined yield dextran preparations that are polydisperse and may, in certain cases,^{42,50} contain structurally distinct dextrans. Relatively homogeneous fractions have been obtained from several partially degraded dextran preparations by submitting them to gel-permeation chromatography,⁶⁴⁻⁷⁶ membrane filtration,^{77,78} and electrophoresis,⁷⁹ although few

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- (72) S. A. Barker, B. W. Hatt, J. B. Marsters, and P. J. Somers, *Carbohydr. Res.*, **9**, 373 (1969).
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instances of fractionation of preparations of native dextrans by these procedures appear to have been recorded.^{79a} The problem of fractionating preparations of native dextrans would, in any event, appear to be twofold, requiring for its solution (a) the initial dispersal of the associations of dextran molecules of high particle weight that are formed during the synthetic reaction,^{80,81} and (b) the separation of the individual, molecular species. In practice, this separation might be achieved by submitting the preparation of the native dextran to gel-permeation chromatography in an alkaline buffer solution.

III. STRUCTURES

The discussion of the structural studies in this Section are divided into two parts: the first deals with the chemical elucidation of dextran structures, and the second, with the application of physicochemical measurements to the examination of network structures formed by association of dextran molecules.

To date the chemical structures of dextrans synthesized by *Leuconostoc* species have been the most thoroughly investigated, although an increasing interest is being evinced in the chemical structures of dextrans produced by streptococci, because of their implication in the development of oral disease (see p. 433). Serological tests^{20,30} have indicated that *Lactobacilli* convert sucrose into dextrans; few confirmatory structural studies have, however, been reported.^{21,28}

Network structures formed by dextrans in the presence of aqueous solvents have not, as yet, been investigated in a systematic manner. The conclusions contained in the second part of this Section are, therefore, based upon experience with a limited number of randomly selected dextrans and, as such, should be accepted with due reservation.

The presentation of the data in this Section has been influenced by the fact that the structure of a dextran is determined primarily by the strain of bacterium that produces it, and not by the species or genus to which the micro-organism belongs. A number of bacteria elaborate water-

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(79) K. Zakshevsky, Z. Mai, and K. Muravsky, *Biokhimiya*, **22**, 596 (1957).

(79a) M. Kobayashi, K. Shishido, T. Kikuchi and K. Matsuda, *Agr. Biol. Chem. (Tokyo)*, **37**, 357 (1973).

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(81) E. Newbrun, R. Lacy, and T. M. Christie, *Arch. Oral Biol.*, **16**, 863 (1971).

soluble and water-insoluble dextrans simultaneously.²¹ These dextran preparations are commonly differentiated by the suffixes *S* (water-soluble), and *L* (less-soluble), and will be so identified in this Chapter.

1. Chemical Structures

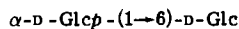
a. The Types of Linkages.—(i) Some Preliminary Experiments. In response to a need to characterize a large number of native dextrans rapidly, Jeanes and her associates²¹ developed several procedures, based on measurements of optical rotations and infrared spectra, and on periodate-oxidation reactions, in order to examine the types and proportions of the different *D*-glucosidic linkages in dextrans. Most of these procedures are still employed during the preliminary examination of dextran structures.^{23,35,82-86} Limitations inherent in each of the procedures referred to, however, preclude the obtaining of wholly reliable data from experiments in which they are employed; for this reason, the results of such experiments will not be discussed here. Detailed descriptions of the procedures may be found in articles by Neely⁶ and Ricketts,⁸ or in the references cited therein. A method for rapidly estimating the numbers of unsubstituted primary hydroxyl groups (effectively, the degree of branching) in clinical dextrans has also been reported.⁸⁶

(ii) Acid Hydrolysis and Acetolysis. The types and configurations of the linkages in a large number of dextrans have been determined by characterizing disaccharide fragments obtained by partial, acid hydrolysis and acetolysis of dextrans.

Partial acid-hydrolyzates of dextrans invariably contain^{28,37,87-92} the

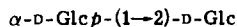
- (82) M. Černý and S. Jaroskiv, *Monatsh.*, **90**, 157 (1959).
- (83) A. S. Saenko, A. B. Livshits, T. V. Polushina, and E. L. Rozenfel'd, *Dokl. Akad. Nauk SSSR*, **157**, 723 (1964).
- (84) Y. Suzuki and K. Uchida, *Ber. ÖHara Inst. Landwirtsch. Biol., Okayama Univ.*, **14**, 1 (1967).
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- (86) J. Holló, E. László, and A. Hoschke, *Periodica Polytech.*, **12**, 277 (1968).
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- (90) B. A. Lewis, M. J. S. Cyr, and F. Smith, *J. Org. Chem.*, **33**, 3139 (1968).
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- (92) E. J. Bourne, R. L. Sidebotham, and H. Weigel, *Carbohydr. Res.*, **22**, 13 (1972).

disaccharide isomaltose (1). In addition, traces of kojibiose⁹² (2), nigerose⁹⁷ (3), and maltose⁹⁹ (4) have been found in acid hydrolyzates



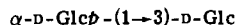
Isomaltose

1



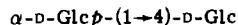
Kojibiose

2



Nigerose

3



Maltose

4

of some highly branched dextrans. The failure to isolate secondary-linked disaccharides from acid hydrolyzates of most dextrans, even though the results of preliminary structural analyses suggested that dextrans usually contain secondary linkages,²¹ was attributed to the fact that secondary D-glucopyranosidic linkages are inherently more acid-labile than (1→6)-D-glucosidic linkages.⁹³⁻⁹⁵ For some, and possibly all, dextrans, the failure may also be related to the fact that the majority of the secondary linkages join residues within acid-labile,^{96,97} terminal, nonreducing sequences in the dextran molecules.⁹⁸⁻¹⁰¹

Fujimoto and coworkers⁹⁵ determined that the order of stabilities to acid hydrolysis of D-glucopyranosidic linkages is reversed under the conditions of acetolysis; this may be due to steric factors affecting the acetylum ion.¹⁰² In consequence, acetolytic, degradative procedures have enabled secondary-linked disaccharide fragments to be obtained from a large number of native dextrans. The α-D-linked disaccharides obtained by acetolysis and deacetylation of a cross-section of dextrans are listed in Table II. The results may be seen to complement data from periodate

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TABLE II
Disaccharides Obtained by Acetolysis of Some Dextrans

Dextran from	α -Glucobioses				References
	Kojibiose (1 \rightarrow 2)	Nigerose (1 \rightarrow 3)	Maltose (1 \rightarrow 4)	Isomaltose (1 \rightarrow 6)	
<i>L. mesenteroides</i>					
NRRL B-512		+		+	74
B-523	+	+		+	103
B-742L		+	+	+	103
B-1149	+	+		+	103
B-1298	+	+		+	90
B-1299	+	+		+	92,103,104
B-1307		+		+	104,105
B-1355		+		+	106
B-1375 ^a		+		+	103
B-1397	+	+		+	107
B-1399	+	+		+	103
B-1415		+	+	+	89
B-1416		+	+	+	89
B-1420		+	+	+	103
B-1424	+	+		+	103,108
strain SF-4		+		+	22,85
44v-2		+		+	22,85
<i>S. bovis</i>					
strain I		+			109
<i>S. mutans</i>					
strain GS 5		+		+	110
<i>Ingbrill</i>	+	+	+	+	37
OMZ 51	+	+		+	37
<i>S. sanguis</i>					
ATCC 10558	+	+		+	37
<i>S. viridans</i> var.					
NRRL B-1351	+	+		+	103
<i>Streptobacterium</i> <i>dextranicum</i> ^b					
NRRL B-1254L		+	+	+	103

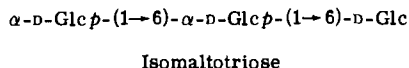
^a Reported to be *L. dextranicum*.²¹

^b Subgenus of *Lactobacillus*.

- (103) H. Suzuki and E. J. Hehre, *Arch. Biochem. Biophys.*, **104**, 305 (1964).
 (104) K. Matsuda, H. Watanabe, K. Fujimoto, and K. Aso, *Nature*, **191**, 278 (1961).
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oxidation and methylation experiments [see Tables V (p. 383) and VI (p. 384)]; and confirm some predictions¹¹¹ founded upon measurements of optical rotations of dextrans in cuprammonium solution. The general occurrence of nigerose (3) is particularly intriguing, and has formed the basis for a suggestion¹⁰³ that dextranucrases (see p. 418) may have some innate ability to transfer D-glucopyranosyl groups to hydroxyl groups at both C-3 and C-6 of D-glucopyranosyl residues.

(iii) **Proton Magnetic Resonance Spectroscopy.** Investigations of the proton magnetic resonance (p.m.r.) spectra of dextrans were initiated by Paseka and Cragg,¹¹² who recorded the spectra of *O*-deuterated *Leuconostoc mesenteroides* NRRL B-512 and NRRL B-742 dextrans in deuterium oxide. Assignment of the bands in these spectra was facilitated by comparison with the p.m.r. spectrum of *O*-deuterated isomaltotriose (5) in deuterium oxide.¹¹²



5

The spectrum of the almost-unbranched NRRL B-512 dextran (see Table VI, p. 384) showed significant bands centered at τ 6.40 (assigned to protons on C-5 and C-6), 6.20 (protons on C-2, C-3, and C-4), 5.30 (DOH), and 5.05 [protons on C-1 involved in the (1 \rightarrow 6)- α -D-glucopyranosidic linkages]. In addition to these bands, the spectrum of NRRL B-742 dextran showed a significant band at τ 4.70. As the NRRL B-742 dextran contains \sim 30% of (1 \rightarrow 3)-linked D-glucopyranosyl residues,²¹ this signal was attributed to protons on the anomeric carbon atoms involved in (1 \rightarrow 3)- α -D-glucopyranosidic linkages. Moreover, the ratio of the areas of the bands at τ 5.05 and 4.70 was found to correspond closely to the ratio of the proportions of (1 \rightarrow 6)- and (1 \rightarrow 3)-linked D-glucopyranosidic residues in NRRL B-742 dextran as determined by periodate oxidation.²¹ On the basis of this observation, it was suggested that p.m.r. spectroscopy might provide a rapid, and general, method for determining the proportions of secondary linkages in dextrans.

The proportions of secondary-linked D-glucopyranosyl residues in several (1 \rightarrow 3)-branched dextrans have been determined from analyses of p.m.r. spectra (see Table III). In each study, the value obtained compared favorably with the proportion of secondary-linked residues

(111) T. A. Scott, N. N. Hellman, and F. R. Senti, *J. Amer. Chem. Soc.*, **79**, 1179 (1957).

(112) W. H. Paseka and L. H. Cragg, *Can. J. Chem.*, **41**, 293 (1963).

TABLE III
Percentages^a of Secondary-Glucosidic Linkages in Some Dextrans

<i>Dextran</i>	<i>Secondary-linked D-glucopyranosyl residues (%)</i>	<i>References</i>
<i>L. mesenteroides</i>		
NRRL B-742	30	112
NRRL B-1355	33	113
NCIB 2706	20	114
<i>S. mutans</i>		
strain <i>Ingbritt</i>	28	37
OMZ 51	17-21	37

^a Calculated from proton magnetic resonance spectra.

calculated from other data. The major bands in the p.m.r. spectra of two (1→2)-branched dextrans⁹² were, however, insufficiently resolved to permit the proportions of the secondary linkages to be determined. The poor resolutions were attributed to the facts that (a) the dextrans have low solubilities, or give turbid solution in deuterium oxide, or both, and (b) the signals from protons on the anomeric carbon atoms associated with the (1→6)- and (1→2)- α -D-glucopyranosidic linkages have similar chemical shifts (see Table IV); this presumably reflects the similar electronegativities of these C-1 substituents.¹¹³

The resolution of the spectra of dextrans having low solubility in deuterium oxide might, conceivably, be improved by recording the spectra in sodium deuteroxide, or deuterated methyl sulfoxide. Com-

TABLE IV
Chemical Shifts of Protons on the Anomeric Carbon Atoms Associated with α -D-Glucopyranosidic Linkages in Some D-Glucans

<i>Glucopyranosidic linkage</i>	<i>Chemical shift (τ)^a</i>	<i>References</i>
1 → 4	4.62	113
1 → 3	4.73	112,113
1 → 2	4.89	92
1 → 6	5.07	113

^a Corrected to DOH (at room temperature) equal to τ 5.30.

(113) C. A. Glass, *Can. J. Chem.*, **43**, 2652 (1965).

(114) D. A. Rees, N. G. Richardson, N. J. Wright, and Sir E. (L.) Hirst, *Carbohydr. Res.*, **9**, 451 (1969).

parison of the chemical shifts of signals from protons on the anomeric carbon atoms involved in α -D-glucopyranosidic linkages in polysaccharides (see Table IV) suggested, however, that the procedure now used is likely to be effective for determining the proportions of secondary linkages in only (1 \rightarrow 4)- and (1 \rightarrow 3)-linked dextrans.

(iv) **Periodate Oxidations.** A number of degradative procedures that utilize the technique of periodate oxidation have been used to determine the nature and proportions of the differently linked D-glucopyranosyl residues in dextrans. Jeanes and her associates^{115,116} developed a method for analyzing the types and proportions of the D-glucopyranosidic linkages in native dextrans from the amounts of periodate ion reduced, and formic acid produced, during the periodate oxidation. A detailed description of this procedure may be found elsewhere.^{115,116} This method of analysis has been used for the rapid, initial characterization of a large number of dextrans.^{21,23,37,82,83,85,116} However, the fact that certain, differently linked, D-glucopyranosyl residues may only be determined collectively (because they react with equal amounts of periodate ion) limits the use of the method where precise data are required.

More-exact structural information may be gained by examining fragments derived from periodate-oxidized dextrans. Aldehydes, some of which are characteristic of structural units in the parent dextran, have been obtained by hydrolyzing periodate-oxidized dextrans with acid.^{22,23,85,117} In this scheme of degradation, D-glucose and D-erythrose, are, for example, characteristic of unoxidized [usually (1 \rightarrow 3)-linked] and (1 \rightarrow 4)-linked D-glucopyranosyl residues, respectively. A method for indirectly estimating these two fragments (as their respective radio-labelled aldonic acids by an isotope-dilution technique) in the acid hydrolyzates of periodate-oxidized dextrans was devised by Moyer and Isbell,¹¹⁷ and used in calculating the proportions of (1 \rightarrow 3)- and (1 \rightarrow 4)-linked D-glucopyranosyl residues in five clinical dextrans.¹¹⁷ By this procedure, *Leuconostoc mesenteroides* NRRL B-512 and NRRL B-1375 clinical dextrans were found to contain, respectively, 4 and 10% of (1 \rightarrow 3)-linked, and 1% each of (1 \rightarrow 4)-linked residues. The presence of (1 \rightarrow 4)-linked residues in these clinical dextrans was unexpected, as this structural feature appears to be absent from the native dextrans (see Tables II, p. 379; V, p. 383; and VI, p. 384). In view of this discrepancy, it is conceivable that the D-erythrose in the hydrolyzates of the periodate-oxidized clinical dextrans arose from incompletely oxidized terminal, non-

(115) A. (R.) Jeanes and C. A. Wilham, *J. Amer. Chem. Soc.*, **72**, 2655 (1950).

(116) J. C. Rankin and A. (R.) Jeanes, *J. Amer. Chem. Soc.*, **76**, 4435 (1954).

(117) J. D. Moyer and H. S. Isbell, *Anal. Chem.*, **29**, 1862 (1957).

TABLE V

Types and Percentages of Differently Linked D-Glucopyranosyl Residues in Some Dextrans, as Determined by Smith Linkage-analysis

Dextran	D-Glucopyranosyl residues (%); linked				References
	(1 → or 1 → 6)	(1 → 4)	(1 → 3)	(1 → 2)	
<i>L. mesenteroides</i>					
NRRL B-512	95		5		119
B-523S	93	3	4		119
B-742S	64	8	28		119
B-742L	95	4	trace		119
B-1064	95	2	3		119
B-1299S	56		7	36	92
B-1299L	49		19	32	92
B-1355S	53		47		119
B-1375 ^a	84.5	1	14.5		121
B-1415	87	12.5	0.5		89
B-1416	83	7	10		89
IFO 12370	94		6		122
strain SF4	95		5		85
strain 44	71		29		85
<i>S. mutans</i>					
OMZ 176	16		84		123
Ingbritt A	37.5		62.5		123a
<i>S. sanguis</i>					
strain 804	52		48		123
Tibi complex ^b	90	1.5	8.5		28

^a Also reported as *L. dextranicum*.²¹

^b *Lactobacillus brevis*/Saccharomyces cerevisiae.

reducing, or (1→6)-linked residues. Quantitative determinations^{22,85} of fragments from periodate-oxidized dextrans from *L. mesenteroides* strains SF-4 and 44 v-2 established that 4 and 29%, respectively, of the linkages in these dextrans are of the (1→3) type.

Difficulty is sometimes experienced in hydrolyzing periodate-oxidized polysaccharides with acid, because they contain stable, anhydro rings.¹¹⁹ If, however, the periodate-oxidized polysaccharide is first reduced to its corresponding polyalcohol, fragments characteristic of structural units in the parent polysaccharide may readily be obtained from the polyalcohol on hydrolysis with dilute acid.¹¹⁸ When this improved degradative

- (118) (a) I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, **5**, 361 (1965); (b) **5**, 377 (1965); see also G. G. S. Dutton, *Advan. Carbohydr. Chem. Biochem.*, **28**, 11 (1973), especially pp. 98-100.

TABLE VI
Types and Percentages of Differently Linked D-Glucopyranosyl Residues
in Some Dextran, as Determined by Methylation Analysis

D-Glucopyranosyl residues (%); linked								Unit chain-length	References
Dextran	1 →	1 → 6	1 → 3	1 → 2,6	1 → 3,6	1 → 4,6			
<i>L. mesenteroides</i>									
NRRL B-512	4	91			5		22.5	124	
B-512	4	91			4.5		23	125	
B-742S ^{a,b}	+	+			+		2.5	97	
B-742L ^a	+	+			+	+	—	97	
B-1298	20	53	1	12	8		4.7	54	
B-1299S ^c	33	26	7	34			3	92	
B-1299L ^c	29	24	15	32			3.3	92	
B-1355S ^d	7	46	40		7		15	97	
B-1375 ^e	17	57			26		4.7	87	
B-1397	20	61	2	14	4		5.2	120	
B-1415	13.5	76				11	8.3	89	
B-1416	15	68				17	6.2	89	
NCIB 2706		85			15		6.7	114	
<i>S. mutans</i>									
strain E. 49	20	52	8 ^f		20		5	36	
GS-5	17	67			16.5		6	110	
OMZ 176	5	40	49.5		5.5		19	126	
Ingbritt A	10	27	51		12		9	123a	
<i>S. sanguis</i>									
strain 804	15	52	18		15		6.7	126	
Tibi complex ^g	5	90	+		5		20	28	

^a Quantitative data unpublished.
derived from data in Refs. 97 and 119.
Saccharomyces cerevisiae.

^b May contain some tetra-*O*-substituted residues.
^c Also reported as *L. dextranicum*.²¹

^c See also, Ref. 125a.

^f Residues (1 → 4)-linked.

^d Analysis de-
^g *Lactobacillus brevis*/

scheme is applied to dextrans,¹¹⁹ D-glyceraldehyde, D-glucose, and erythritol become, respectively, the characteristic fragments of (1→2)-, unoxidized [usually (1→3)-linked], and (1→4)-linked D-glucopyranosyl residues, whereas glycerol or glycolaldehyde may be used to identify terminal, nonreducing, or (1→6)-linked residues. The structures of several *Leuconostoc*^{22,85,89,92,119-122} and streptococcal^{28,35,36,47,123,123a} dextrans were examined by this degradative procedure; the results of some quantitative experiments are given in Table V, and an interesting feature is the incidence of unoxidized D-glucopyranosyl residues in all of the dextrans. Acetolysis experiments (see Table II, p. 379) indicated that these residues are (1→3)-linked.

(v) **Methylation Studies.** The nature and proportions of the differently linked D-glucopyranosyl residues in several native (see Table VI) and clinical^{125,127,128} dextrans were determined by the technique of methylation. It may be seen from the results in Table VI that all of the native dextrans have branched structures in which the branching occurs through tri-*O*-substituted D-glucopyranosyl residues. Seven of the preparations listed contain two different types of secondary linkage, suggesting that they may be composed of at least two, structurally distinct, glucans. Of particular interest are (a) the presence of (1→3)-linked residues in all of the dextran preparations, and (b) the fact that, in some dextrans, these are not branching residues. The finding of 8% of (1→4)-linked residues in a preparation of *Streptococcus mutans* E.49 dextran³⁶ may reflect the presence of a glycogen-like contaminant in the preparation. The double-branching residues in *Leuconostoc mesenteroides*

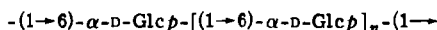
- (119) W. J. Sloan, B. H. Alexander, R. L. Lohmar, I. A. Wolff, and C. E. Rist, *J. Amer. Chem. Soc.*, **76**, 4429 (1954).
- (120) H. Miyaji, A. Misaki, and M. Torii, *Carbohydr. Res.*, **31**, 277 (1973).
- (121) D. Abbott and H. Weigel, unpublished results.
- (122) A. Misaki and S. Kanamaru, *Agr. Biol. Chem.* (Tokyo), **32**, 432 (1968).
- (123) B. Guggenheim, *Helv. Odont. Acta*, **14**, 89 (1970).
- (123a) J. K. Baird and D. C. Ellwood, *Biochem. J.*, **127**, 88P (1972).
- (124) J. W. Van Cleve, W. C. Shaefer, and C. E. Rist, *J. Amer. Chem. Soc.*, **78**, 4435 (1956).
- (125) B. Lindberg and S. Svensson, *Acta Chem. Scand.*, **22**, 1907 (1968).
- (125a) M. Kobayashi, K. Shishido, T. Kikuchi, and K. Matsuda, *Agr. Biol. Chem.* (Tokyo), **37**, 2763 (1973).
- (126) M. Ceska, K. A. Granath, B. Norrman, and B. Guggenheim, *Acta Chem. Scand.*, **26**, 2223 (1972).
- (127) J. K. N. Jones and K. C. B. Wilkie, *Can. J. Biochem. Physiol.*, **37**, 377 (1959).
- (128) H. S. Isbell, H. L. Frush, B. H. Bruckner, G. N. Kowkabany, and G. Wampler, *Anal. Chem.*, **29**, 1523 (1957).

NRRL B-742 dextran may be artifactual structures, resulting from incomplete methylation of this highly branched polysaccharide.⁹⁷

Dextrans have been methylated most frequently by the Haworth method,^{28,38,54,87,127} and by procedures that employ sodium and methyl iodide in liquid ammonia.^{89,92,124,127,128} Rapid methylation of dextrans has been achieved^{86,101,110,123a} through the use of the Hakomori procedure,¹²⁹ which utilizes methyl sulfoxide as solvent for the dextran. Careful control of the reaction temperature would, however, appear to be essential when a dextran is methylated in this solvent, as hot methyl sulfoxide has been reported to depolymerize native dextrans.¹³⁰

The use of gas-liquid chromatographic and mass-spectroscopic techniques in combination have greatly facilitated the separation and identification of fragments from *O*-methylated dextrans. For detailed descriptions of these procedures, the reader is referred to a series of papers by Lindberg and associates.¹³¹⁻¹³⁴

b. Structural Segments.—(i) **Acid Hydrolysis and Acetolysis.** These complementary degradative procedures (see p. 377) have yielded oligosaccharides that provided evidence of structural segments in several dextrans. Isomaltose (1, see p. 378) and higher homologs containing up to eight or nine residues are usually obtained on partially hydrolyzing dextrans with acid.^{37,87-92} It may be presumed that the homologs of isomaltose arise from sequences of (1→6)-linked α -D-glucopyranosyl residues that form the skeletal chains of the dextrans, as in structure 6.



6

In comparison, higher oligosaccharides have as yet been obtained, following deacetylation of the products, in acetolyzates of only a few dextrans. The trisaccharides *O*- α -D-glucopyranosyl-(1→6)-*O*-[α -D-glucopyranosyl-(1→2)]-D-glucose (7) and *O*- α -D-glucopyranosyl-(1→2)-*O*- α -D-glucopyranosyl-(1→6)-D-glucose (8) were isolated from an acetolyzate of *Leuconostoc mesenteroides* NRRL B-1298 dextran.⁹⁰ This finding, and the absence of the isomeric trisaccharide *O*- α -D-glucopyranosyl-(1→6)-*O*-

(129) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

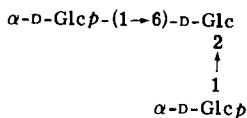
(130) W. Kaufmann and K. Bauer, German Pat. 1,007,951 (1957); *Chem. Abstr.*, **54**, 16,757 (1960).

(131) H. Björndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967).

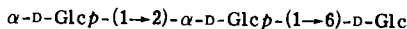
(132) H. Björndal, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, **21**, 1801 (1967).

(133) H. Björndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **15**, 339 (1970).

(134) H. Björndal, C. G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem.*, **9**, 610 (1970).

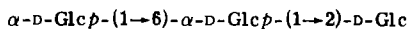


7

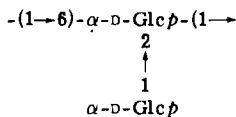


8

α -D-glucopyranosyl-(1 \rightarrow 2)-D-glucose (9), indicated that the branches associated with the (1 \rightarrow 2)-branch points in this dextran may be exclusively 2-O- α -D-glucopyranosyl groups, as shown in partial structure 10.

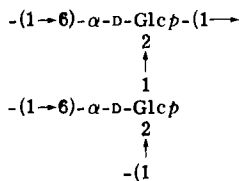


9

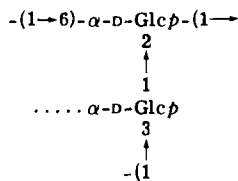


10

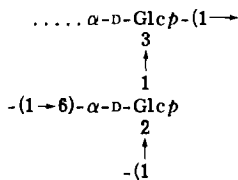
On acid hydrolysis, a second trisaccharide fraction from NRRL B-1298 dextran⁹⁰ gave D-glucose, kojibiose (2, see p. 378), and nigerose (3) and, consequently, could only have arisen from segments in the dextran where two secondary-linked α -D-glucopyranosyl residues are adjacent (see structures 11 to 14).



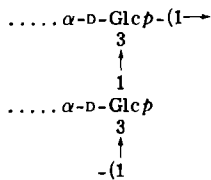
11



12

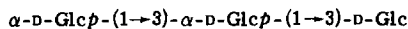


13



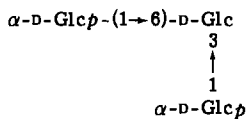
14

Similar branched or linear segments (or both; see structure 14) are also present in *Leuconostoc mesenteroides* NRRL B-1307 dextran,¹⁰⁵ as nigerotriose (15) and the branched trisaccharide *O*- α -D-glucopyranosyl (1 \rightarrow 6)-*O*-[α -D-glucopyranosyl-(1 \rightarrow 3)]-D-glucose (16) were obtained following acetolysis of the dextran.



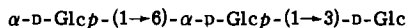
Nigerotriose

15



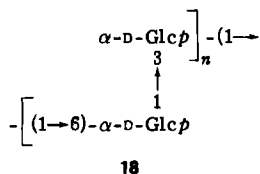
16

O- α -D-Glucopyranosyl-(1 \rightarrow 6)-*O*- α -D-glucopyranosyl-(1 \rightarrow 3)-D-glucose (17) was the only trisaccharide isolated from an acetolyzate of *Leu-*



17

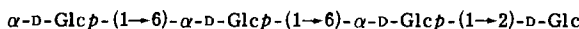
conostoc mesenteroides NRRL B-1355S dextran.¹⁰⁶ The failure to isolate the isomeric, branched trisaccharide 16 is particularly significant, because it suggested that most of the (1 \rightarrow 3)-linked D-glucopyranosyl residues are present in linear segments of the dextran molecule. The absence from the acetolyzate of derivatives of nigerotriose (15), and the fact that this dextran contains a remarkably high proportion of (1 \rightarrow 3)-linked glucopyranosyl residues [see Tables V (p. 383) and VI (p. 384)], further suggested that some linear segments in the dextran may be composed of sequences of alternating (1 \rightarrow 3)- and (1 \rightarrow 6)-linked α -D-glucopyranosyl residues, as in structure 18.



18

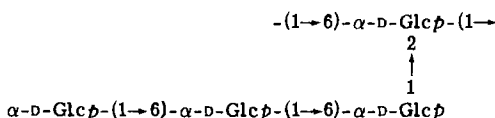
On acetolysis and deacetylation, *Leuconostoc mesenteroides* NRRL B-1299S dextran¹³⁵ gave, amongst other oligosaccharides, trisaccharide 9 (135) R. L. Sidebotham and H. Weigel, unpublished results.

and the homologous tetrasaccharide *O*- α -D-glucopyranosyl-(1 \rightarrow 6)-*O*- α -D-glucopyranosyl-(1 \rightarrow 6)-*O*- α -D-glucopyranosyl-(1 \rightarrow 2)-D-glucose (19). As



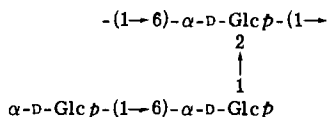
19

all the (1→2)-linkages in this dextran are branch-linkages (see Table VI, p. 384), tetrasaccharide **19** must have arisen from a segment in which the branch chain consists of (at least) a 2-*O*- α -isomaltotriosyl group, as in partial structure **20**.



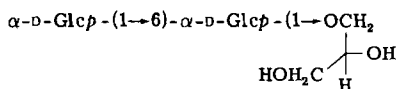
20

The isomeric trisaccharides **7**, **8**, and **9** have, in addition, been obtained by acetolysis and deacetylation of *L. mesenteroides* NRRL B-1397 dextran,¹⁰⁷ and provide evidence that some branches are composed of 2-*O*- α -isomaltosyl (or larger) groups, as shown in structure **21**.

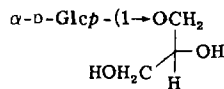


21

(ii) **Smith Degradation.** Dextrans generally contain (1→3)-linked α -D-glucopyranosyl residues (see Table II, p. 379), and as these residues are resistant to oxidation by periodate ion, their distribution in dextran molecules may be investigated by Smith degradation.^{118a} The Smith procedure of controlled degradation by sequential periodate oxidation, reduction, and hydrolysis with dilute acid has been used to isolate fragments characteristic of structural segments in several dextrans.^{28,54,100,106,122} On Smith degradation, *Leuconostoc mesenteroides* NRRL B-1298 and NRRL B-1375 dextrans^{54,100} yielded both 1-O- α -isomaltosylglycerol (**22**) and 1-O- α -D-glucopyranosylglycerol (**23**). As the

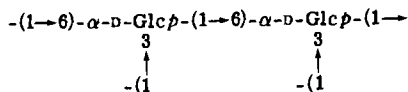
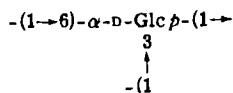


22



23

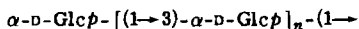
(1→3)-D-glucopyranosidic linkages in these dextrans form branch points (see Table VI, p. 384), fragment **22** must have arisen from segments in the dextrans where two branching, (1→3)-linked D-glucopyranosyl residues are adjacent (as in structure **24**), and fragment **23**, from segments containing an isolated branching residue (as in structure **25**). The

**24****25**

proportion of isomaltosylglycerol (**22**) obtained from NRRL B-1375 dextran¹⁰⁰ indicated that at least 17% of all the branches in the dextran are joined to contiguous, (1→6)-linked α -D-glucopyranosyl residues.

In contrast, 1-O- α -D-glucopyranosylglycerol (**23**) was the largest fragment characterized from Smith-degraded *L. mesenteroides* NRRL B-1355S and strain IFO 12370, and Tibi-complex dextrans.^{28,106,122} Segment **24** cannot, therefore, be a significant structural feature of these dextrans.

The absence of derivatives of nigero-oligosaccharides (**26**) from amongst the fragments from these Smith-degraded dextrans suggests that sequences of (1→3)-linked D-glucopyranosyl residues may generally (but not exclusively^{105,123,136}) be absent from dextran molecules.

**26**

(iii) **Enzymic Degradations.** In comparison to the starch-glycogen group of D-glucans, few enzymic degradational studies on dextrans have thus far been documented. Most of the structural studies have involved enzymes that hydrolyze the (1→6)- α -D-glucopyranosidic linkages of the skeletal chains, although enzyme preparations that cleave (1→3)-intra-chain and (1→4)-branch α -D-glucopyranosidic linkages have been reported.

(136) B. Guggenheim and R. Haller, *J. Dent. Res.*, **51**, 394 (1972).

Dextranases [(1→6)- α -D-glucan 6-glucanohydrolases: EC.3.2.1.11] are enzymes that specifically hydrolyze the (1→6) linkages in dextrans. Dextranase production has been induced in fungi and bacteria by growing the micro-organisms on dextrans,¹³⁷⁻¹⁴⁶ ketodextrans,¹⁴⁷ or ester derivatives of isomaltose¹⁴⁸ (1). Related enzymes are present in mammalian tissues,^{138,149-152} and have been found in the coleoptiles of *Avena*.¹⁵³ The dextranase-induction process has been discussed by Reese and coworkers.¹⁴⁸

Exodextranases catalyze a stepwise hydrolysis of the dextran molecule to yield D-glucose and a residual dextran; they have been extracted from cultures of species of *Bacillus*,¹⁴² *Bacteroides*,¹³⁸ and *Lactobacillus bifidus*.¹⁵⁴ These enzymes have not, however, been utilized in structural studies. Endodextranases have been isolated from exocellular fluids or cell extracts (or both) of the fungi *Penicillium funiculosum*,¹⁴⁰ *P. lilaci-*

- (137) H. M. Tsuchiya, A. (R.) Jeanes, H. M. Bricker, and C. A. Wilham, *J. Bacteriol.*, **64**, 513 (1952).
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- (140a) M. Sugiura, A. Ito, T. Ogiso, K. Kato, and H. Asano, *Biochim. Biophys. Acta*, **309**, 357 (1973).
- (141) (a) J. C. Janson and J. Porath, *Methods Enzymol.*, **8**, 615 (1966); (b) J. C. Janson, Dissertation, Uppsala, 1972.
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- (151) B. Ingelman, *Acta Acad. Regiae Sci. (Uppsala)*, **12**, 17 (1969).
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- (153) A. N. J. Heyn, *Science*, **167**, 874 (1970).
- (154) R. W. Bailey and A. M. Robertson, *Biochem. J.*, **82**, 272 (1962).

num,¹⁴⁰ *P. luteum*,¹⁴⁴ and *Aspergillus carneous*,¹⁵⁵ and of the bacteria *Lactobacillus bifidus*¹³⁹ and *Cytophaga*.¹⁴¹ From these sources, preparations that randomly cleave the dextran molecule to oligosaccharides have been used in structural studies.^{74,139,140a,156-159}

Detailed studies of the action of exocellular dextranases produced by two *Penicillium* species,^{156,157} and *Lactobacillus bifidus*¹³⁹ on *Leuconostoc mesenteroides* NRRL B-1375 and NRRL B-1415 dextrans have been reported by Weigel and associates. In addition to small proportions of D-glucose, the enzyme hydrolyzates contained isomaltose (1, p. 378), isomaltotriose (5, p. 380), and homologous series of limit dextrans. Each limit dextrin possessed the unhydrolyzed branch-linkage of the parent dextran (see Table VI, p. 384) joined to a homolog of isomaltose (1). The branch chains in these oligosaccharides, moreover, appeared to consist exclusively of single D-glucopyranosyl groups. This finding, in conjunction with action patterns proposed for the dextranases^{135,160} (see Fig. 1), indicated that the limit dextrans arose from

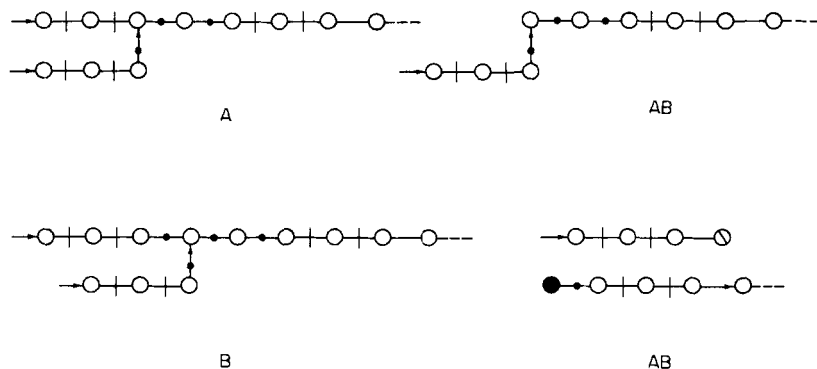
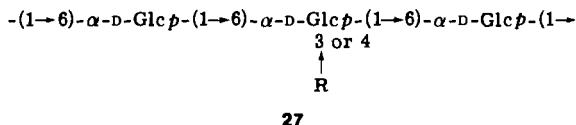


FIG. 1.—The Actions of *P. funiculosum* (A) and *P. lilacinum* (B) Endodextranases on Branched and Linear Segments of Dextrans. [—○ = (1→6)-linked α-D-glucopyranosyl residue; ○ = secondary-linked α-D-glucopyranosyl residue; ⊙ = terminal, D-glucose residue; ● = terminal, D-glucopyranosyl group; —, —○, —● = linkages resistant to hydrolysis; + = alternative points of hydrolysis that give rise to stable oligosaccharides.]

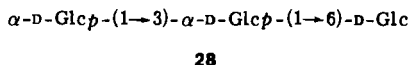
- (155) N. Hiraoka, J. Fukumoto, and D. Tsuru, *J. Biochem. (Tokyo)*, **71**, 57 (1972).
- (156) E. J. Bourne, D. H. Hutson, and H. Weigel, *Biochem. J.*, **86**, 555 (1963).
- (157) D. Abbott and H. Weigel, *J. Chem. Soc.*, 821 (1966).
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- (159) E. T. Reese and F. W. Parrish, *Biopolymers*, **4**, 1043 (1966).
- (160) D. H. Hutson and H. Weigel, *Biochem. J.*, **88**, 588 (1963).

parts of the two dextrans (as in partial structure 27) where the branches are mainly α -D-glucopyranosyl groups or α -isomaltotriosyl groups, or both. Additional evidence, indicating that most of the branches in these dextrans are α -D-glucopyranosyl groups, is discussed on p. 409.

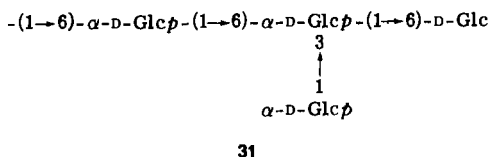
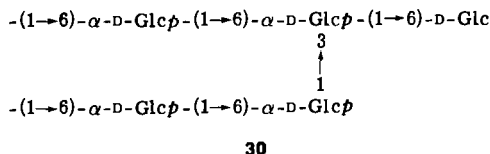
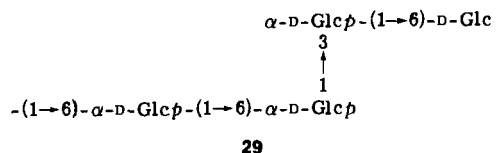


where R = α -D-Glc p-1 or α -D-Glc p-(1 \rightarrow 6)- α -D-Glc p-(1 \rightarrow 6)- α -D-Glc p-1.

Treatment of two acid-degraded dextrans with exocellular, fungal dextranases has also been reported.^{74,158} On incubation with *Penicillium funiculosum* dextranase,⁷⁴ acid-degraded *Leuconostoc mesenteroides* NRRL B-512 dextran yielded a mixture of oligosaccharides, including nigerose (3) and the trisaccharide *O*- α -D-glucopyranosyl-(1 \rightarrow 3)-*O*- α -D-glucopyranosyl-(1 \rightarrow 6)-D-glucose (28). These oligosaccharides have not

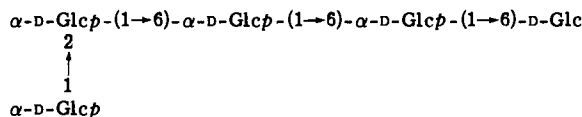


been observed in hydrolyzates of the native dextran.¹³⁵ This fact, in conjunction with the action pattern of *P. funiculosum* dextranase (see Fig. 1) indicates that fragments 3 and 28 must have come from acid-modified, terminal, reducing segments in the degraded dextran. Trisaccharide 28, for example, might have arisen from segment 29, 30, or 31. As

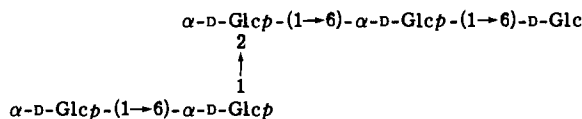


all the (1→3)-linkages in native NRRL B-512 dextran form branch points (see Table VI, p. 384), some branches in the native dextran might thus be composed of three or more α -D-glucopyranosyl residues (see Table VII, p. 407).

The pentasaccharides O - α -D-glucopyranosyl-(1→2)- O - α -D-glucopyranosyl-(1→6)- O - α -D-glucopyranosyl-(1→6)- O - α -D-glucopyranosyl-(1→6)-D-glucose (32) and O - α -D-glucopyranosyl-(1→6)- O - α -D-glucopyranosyl-(1→2)- O - α -D-glucopyranosyl-(1→6)- O - α -D-glucopyranosyl-(1→6)-D-glucose (33) were identified, amongst other oligosaccharides, in a *Penicillium*

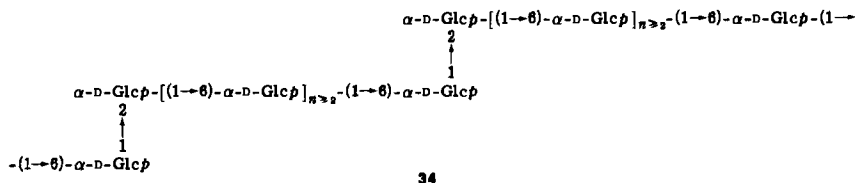


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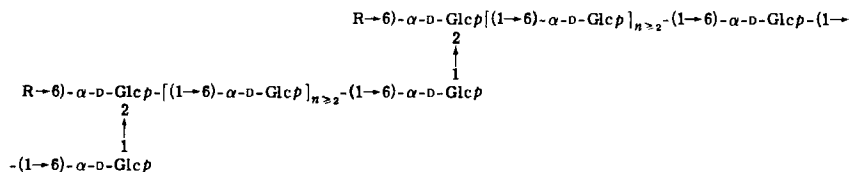
33

lilacinum dextranase hydrolyzate of acid-degraded *Leuconostoc mesenteroides* NRRL B-1299S dextran.¹⁵⁸ Analogous oligosaccharides have not been found in *P. lilacinum* dextranase hydrolyzates of native dextrans,^{156,157} and the authors concluded that pentasaccharides 32 and 33 were fragments from unbranched, acid-modified segments of the degraded dextran, as in partial structure 34. As all the (1→2) linkages in



34

native NRRL B-1299S dextran are branch-linkages (see Table VI, p. 384), the native dextran must contain segments of the kind represented by partial structure 35. Statistical analysis of fragments of low molecular weight obtained by partial hydrolysis of native NRRL B-1299S dextran with acid indicated, moreover, that most of the external branches in this dextran are D-glucopyranosyl groups; this finding suggested that group R in structure 35 will frequently be an α -D-glucopyranosyl group, and, by



extension, that the substituents on some branching residues in NRRL B-1299S dextran are 2-*O*- α -isomaltopentaosyl (or larger) groups.

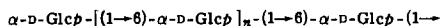
A limitation to the usefulness of fungal endodextranases in investigating dextran structures stems from their inability to degrade highly branched dextrans.^{49,126,140,158} Action patterns that have been proposed for fungal endodextranases in the vicinity of intrachain and branch secondary-linkages (see Fig. 1, p. 392) do, however, indicate that the positions of the secondary linkages may be important in determining the degradability of a dextran: a linear structure containing isolated secondary-linkages should be degraded more readily, to yield smaller fragments overall, than one containing isolated, secondary branch-linkages. Such factors as these are undoubtedly prime considerations in determining the practical value of fungal endodextranases in removing dextrans from sugar-cane or beet juices (see p. 444), in controlling the formation of dental plaque (see p. 440), and in preparing clinical-size dextrans.¹⁶¹

From liver and spleen homogenates Rozenfel'd and associates isolated enzymes that catalyze the stepwise hydrolysis of dextrans and glycogens.¹⁶²⁻¹⁶⁸ A study of the actions of these enzymes, termed dextran-glucosidases [EC 3.2.1.3/11], upon several native and clinical-size dextrans revealed that they sequentially cleave α -D-glucopyranosyl groups from the (1 \rightarrow 6)-linked skeletal chains of dextrans up to the branching points, with the liberation of D-glucose and residual dextrans.¹⁶³⁻¹⁶⁶ The extent of degradation of a dextran is thus determined by (a) its degree of branching, and (b) the lengths of uninterrupted terminal, nonreduc-

- (161) L. J. Novak and G. S. Stoycos, U. S. Pat. 2,841,578 (1958); *Chem. Abstr.*, **52**, 17,627 (1958).
- (162) E. L. Rozenfel'd, A. I. Shubina, and A. A. Kuznetsov, *Dokl. Akad. Nauk SSSR*, **104**, 115 (1955).
- (163) E. L. Rozenfel'd, *Biokhimiya*, **21**, 84 (1956).
- (164) E. L. Rozenfel'd and I. S. Lukomskaya, *Clin. Chim. Acta*, **2**, 105 (1957).
- (165) I. S. Lukomskaya and E. L. Rozenfel'd, *Biokhimiya*, **23**, 261 (1958).
- (166) E. L. Rozenfel'd, *Biokhimiya*, **23**, 635 (1958).
- (167) E. L. Rozenfel'd, *Bull. Soc. Chim. Biol.*, **42**, 1575 (1960).
- (168) E. L. Rozenfel'd, I. S. Lukomskaya, N. K. Rudakova, and A. I. Shubina, *Biokhimiya*, **24**, 1047 (1959).

ing sequences of (1→6)-linked α -D-glucopyranosyl residues.¹⁶⁴ Lowering of the molecular weights by some 18 to 35% was reported^{166,167} when dextran glucosidase preparations were incubated with the clinical dextrans Polyglukan, Polyglukin,* Intradex,† and Macrodex. Calculations based upon the degree of degradation and the proportions of secondary linkages in the clinical dextrans indicated that, on average, the branch chains are composed of two (Polyglukan), three (Macrodex), and six (Intradex and Polyglukin) (1→6)-linked α -D-glucopyranosyl residues, respectively. The same enzymes effected a 20 to 25% lowering in the molecular weights of several unspecified native dextrans;¹⁶⁸ these degrees of hydrolysis are consistent with there being several, long branch-chains in the dextrans.

Degradation of a similar magnitude (38%) was also reported when an acid-degraded *Leuconostoc mesenteroides* NRRL B-512 dextran that contained 95% of (1→6)- α -D-glucopyranosidic linkages was incubated with an exo- α -D-glucan 6-glucanohydrolase [EC 3.2.1.70] isolated from a strain⁶¹ of *Streptococcus mitis*. As it may be assumed that all of the secondary linkages in the degraded dextran form branch points,¹²⁵ some of the branch chains in the dextran would appear to be composed of at least eight (1→6)-linked residues, as in structure 36 ($n = 6$).



36

The extents to which four native dextrans were degraded by the same enzyme preparation were found to correlate with the proportions of (1→6)-linked residues in each dextran.^{168a} Calculations based upon the percentages of secondary linkages in the dextrans, and the degrees of conversion of the dextrans into D-glucose suggest, furthermore, that some branch chains in *L. mesenteroides* NRRL B-742 and NRRL B-1415 dextrans consist of two or more (1→6)-linked α -D-glucopyranosyl residues, and that a number of branches in NRRL B-512F dextran are composed of at least five such residues.

It has been demonstrated^{63,169} that clinical-size dextrans are completely metabolized *in vivo* by liver enzymes. Liver extracts would, therefore, appear to be potential sources of dextran-debranching enzymes. Rozenfel'd and her associates^{22,83,169} examined preparations from homogenized liver, kidney, and spleen, but failed to isolate an enzyme

* *Leuconostoc mesenteroides* strain SF-4 dextran partially hydrolyzed by acid.

† *Leuconostoc mesenteroides* NRRL B-1375 (Birmingham) dextran partially hydrolyzed by acid.

(168a) G. J. Walker and A. Pulkownik, *Carbohydr. Res.*, **29**, 1 (1973).

(169) E. L. Rozenfel'd and I. A. Popova, *Vopr. Med. Khim.*, **8**, 468 (1962).

having debranching activity. Enzyme preparations able to cleave both (1→6)- and (1→3)- α -D-glucopyranosidic linkages in selected dextrans, to yield D-glucose and a residual dextran,^{83,169} were, however, obtained. To explain these results, it was assumed^{22,83} that the enzyme preparations hydrolyze (1→6)- and (1→3)- α -D-glucopyranosidic linkages in linear segments of the dextrans, but not branch linkages.

An enzyme preparation isolated from bovine spleen was employed to examine the structures of dextrans^{28,85,169a} produced by *L. mesenteroides* strains 44B-2 and 54-2A. On incubation with the enzyme preparation, the molecular weights of the two dextrans were decreased by 14 and 12% respectively, and the proportions of (1→3)-linked D-glucopyranosyl residues were changed from 31 to 21%, and from 19 to 2%, respectively. These findings suggest that the dextrans contain two unusual structural features: at least a third of the (1→3)- α -D-glucopyranosidic linkages in strain 44B-2 and almost all the (1→3)-linkages in strain 54-2A may not form branch points, and are situated within terminal, non-reducing sequences in the branch chains or main chain. Alternatively, the polysaccharide preparations might contain branched dextrans in admixture with D-glucans primarily (1→3)-linked (see Ref. 126).

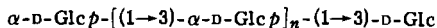
The presence of a high proportion of (1→3)-linked α -D-glucopyranosyl residues in the branch chains of dextrans produced by *L. dextranicum* strain 61-2 and *L. mesenteroides* strains 44B-2 and 44m-2 may also account for the observation that these dextrans, which contain 29–36% of (1→3)-linked residues, were cleaved at a far higher rate by a purified α -D-glucosidase from pig spleen than were *L. mesenteroides* strains SF-4 and 63-1 dextrans, which contain fewer than 5% of (1→3) linkages.^{169b}

A large number of commercial dextranase preparations have been found to cause slow hydrolysis of streptococcal dextrans that contain high percentages of (1→3)-linked D-glucopyranosyl residues.⁴⁹ To explain this, it was suggested^{49,123} that the dextranase preparations might be contaminated with α -glucan 3-glucanohydrolases [EC 3.2.1.59/84], whose production had been induced by the small proportion of (1→3)-linked α -D-glucopyranosyl residues present in the *Leuconostoc* species dextrans that are usually used to stimulate dextranase formation. Guggenheim and Haller^{123,136} demonstrated that commercial *Penicillium* dextranases do, indeed, contain minor proportions of endo- and exo- α -D-glucan 3-glucanohydrolases, and that a number of fungi that elaborate dextranases when cultured on dextrans produce α -D-glucan 3-glucanohydrolases when grown on (1→3)-linked D-glucans.

(169a) M. E. Preobrazhenskaya and E. L. Rozenfel'd, *Biokhimiya*, **35**, 753 (1970).

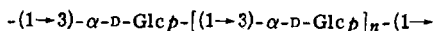
(169b) M. E. Preobrazhenskaya and E. L. Rozenfel'd, *Biokhimiya*, **37**, 974 (1972).

The mixed endo- and exo- α -glucan 3-glucanohydrolases isolated from cultures of a species of *Penicillium*¹²³ and of *Trichoderma harianum* OMZ 779 (Ref. 136) have been employed in studying the structures of the water-insoluble dextrans synthesized by *Streptococcus sanguis* strain 804 and *S. mutans* OMZ 176 that contain unusually high proportions of (1 \rightarrow 3)-linked D-glucopyranosyl residues (see Table VI, p. 384). The OMZ 176 dextran was rapidly hydrolyzed to D-glucose, isomaltose (1, p. 378), nigerose (3), and unidentified, higher oligosaccharides by the *Penicillium* species enzyme;¹²³ from this result, it was concluded that the dextran contains sequences of (1 \rightarrow 3)-linked α -D-glucopyranosyl residues. Both of the dextran preparations were hydrolyzed by *Trichoderma harianum* OMZ 779 α -D-glucan 3-glucanohydrolase.¹³⁶ As this enzyme cleaves polymer chains composed of (1 \rightarrow 3)-linked α -D-glucopyranosyl residues, but not nigerotetraose (37, $n = 2$), to D-glucose and nigerose



37

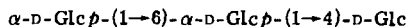
(3, see p. 378) many of the (1 \rightarrow 3)-linked residues must form, within these dextrans, sequences of the kind represented by partial structures 26 or 38 ($n > 4$), or both. Alternatively, the preparations may consist



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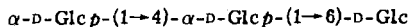
of mixtures of branched dextrans and D-glucans primarily (1 \rightarrow 3)-linked. The extent of the hydrolysis of *Streptococcus sanguis* 804 dextran¹³⁶ further indicated that $\sim 25\%$ of the D-glucopyranosyl residues are (1 \rightarrow 3)-linked.

The enzyme glucoamylase [α -D-glucan 4-glucanohydrolase; EC 3.2.1.3] has a broad substrate-specificity: it catalyzes the hydrolysis of maltose (4, p. 378) and the stepwise removal of α -D-glucopyranosyl groups from the chain-ends of starch and glycogen. In addition, Pazur and associates reported^{170,171} that a preparation from *Aspergillus niger* strain 152 catalyzes the slow hydrolysis of (1 \rightarrow 6)- α -D-linkages in isomaltose (1, p. 378), panose (39), and isopanose (40), and limited reversion-reactions



Panose

39



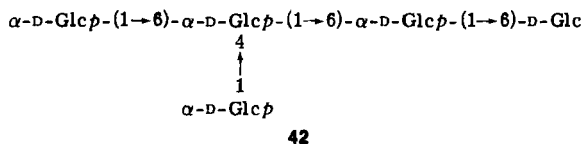
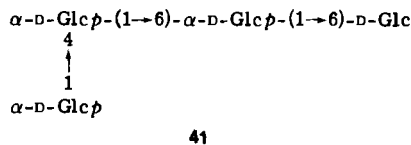
Isopanose

40

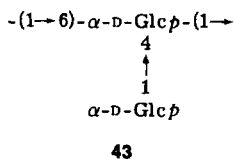
(170) J. H. Pazur and T. Ando, *J. Biol. Chem.*, **235**, 297 (1960).

(171) J. H. Pazur and S. Okada, *Carbohydr. Res.*, **9**, 371 (1967).

when incubated with D-glucose. Abbott and Weigel¹⁵⁷ isolated from the same source a glucoamylase preparation that was essentially free from isomaltose activity, but rapidly hydrolyzed amylose, maltose (4, p. 378), and the (1→4)-linkages of *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (41) and *O*-α-D-glucopyranosyl-(1→6)-[*O*-α-D-glucopyranosyl-(1→4)]-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (42). The same



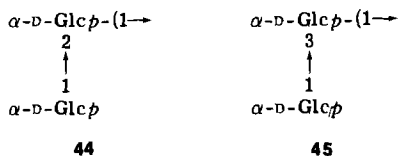
enzyme preparation was found¹⁰⁰ to liberate D-glucose from the (1→4)-branched *Leuconostoc mesenteroides* NRRL B-1415 and NRRL B-1416 dextrans (see Table VI, p. 384). Quantitative determination of the D-glucose [which could have arisen only from hydrolysis of (1→4)-branch linkages] indicated that at least 66% of the branches in NRRL B-1415 dextran, and 33% of the branches in NRRL B-1416 dextran, are composed of 4-*O*-α-D-glucopyranosyl groups, as in partial structure 43.



(iv) **Immunochemical Studies.** Dextrans are able to participate in immunochemical reactions in which the dextran, acting as a soluble antigen, respectively precipitates soluble antidextran, antipneumococcal, or antiteichoic acid antibodies. The magnitude of these precipitin reactions may be gauged by determining the proportion of antibody protein (as nitrogen) in the precipitated dextran-antibody complex. Oligosaccharides, or sugar derivatives (haptens) of suitable structure, are able to inhibit particular dextran-antibody reactions by combining with the antibody molecules. Studies of hapten inhibition-reactions permitted

elucidation of the structural requirements of the combining sites of antibody molecules and, then, the arrangements of the α -D-glucopyranosyl residues within the terminal, nonreducing sequences of a number of native dextrans.

A. Dextran-Antidextran Reactions. Kabat and associates discovered that purified dextrans are antigenic in animals,¹⁷² including man.^{98,173,174} Following injection with dextrans containing various percentages of secondary linkages, most individuals produced antibodies specific against (1 \rightarrow 6)-linked α -D-glucopyranosyl residues.^{174,175} A few individuals produced mixtures of antibodies, the main population being specific to secondary [that is, non-(1 \rightarrow 6)-linked] α -D-glucopyranosyl residues.^{98,172,174} Inhibition studies conducted with oligosaccharides established that the (1 \rightarrow 6)-specific antibodies react against terminal, nonreducing sequences of three to seven (1 \rightarrow 6)-linked, α -D-glucopyranosyl residues.^{172,174,176,177} Non-(1 \rightarrow 6)-specific antibodies produced by one individual to *Leuconostoc mesenteroides* NRRL B-1299S dextran reacted against terminal, nonreducing sequences of two, or three, secondary-linked, α -D-glucopyranosyl residues.¹⁷⁴ In other individuals, non-(1 \rightarrow 6)-specific antibodies to *L. mesenteroides* NRRL B-1299S and NRRL B-1355S dextrans were found to be directed against kojibiosyl (44), and nigerosyl (45) groups, respectively.⁹⁸



The individual abilities of 14 dextrans to precipitate (1 \rightarrow 6)-specific antibodies from four different sera were found to be dependent upon the proportion of (1 \rightarrow 6)-linked D-glucopyranosyl residues in each dextran.¹⁷⁵ On a molar basis, the most effective inhibitors of these precipitin reactions are isomaltotetraose (structure 46, $n = 2$) and isomaltopentaose (structure 46, $n = 3$), from which it was concluded that all of the dextrans studied possessed numerous terminal, nonreducing sequences

(172) R. G. Mage and E. A. Kabat, *J. Immunol.*, **91**, 633 (1962).

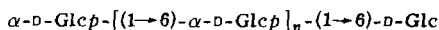
(173) E. A. Kabat and D. Berg, *J. Immunol.*, **70**, 514 (1953).

(174) E. A. Kabat, *J. Amer. Chem. Soc.*, **76**, 3709 (1954).

(175) P. Z. Allen and E. A. Kabat, *J. Amer. Chem. Soc.*, **78**, 1890 (1956).

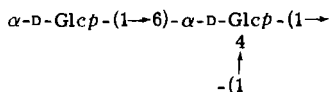
(176) E. A. Kabat, *Bull. Soc. Chim. Biol.*, **42**, 1549 (1960).

(177) S. F. Schlossman and E. A. Kabat, *J. Exp. Med.*, **116**, 535 (1962).

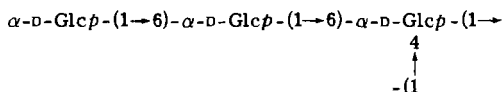


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(branch chains) consisting of at least four or five (1→6)-linked α -D-glucopyranosyl residues (structure 36; $n = 2$ or 3), although the numbers of such sequences were found greatest in the less-highly branched dextrans. In this survey, the behavior of two dextrans was "anomalous." *Leuconostoc mesenteroides* NRRL B-1299S dextran was found to be more effective, and *L. mesenteroides* NRRL B-742L dextran less effective, in precipitating antibodies than could be expected from the proportions of (1→6)-linked D-glucopyranosyl residues in the respective polysaccharides. To explain these differences, it was suggested^{175,176,178} that (a) an unusually large number of branch chains in NRRL B-1299S must be terminated by α -isomalto-oligosaccharidyl groups (as in structure 36, p. 396), possibly because many of the secondary-linked [presumably (1→3)-linked⁹²] residues are present in continuous sequences, and (b) NRRL B-742L dextran might contain numerous segments of the kind represented, for example, by partial structures 43, 47, or 48.



47



48

Quantitative, precipitin curves showed the immunochemical reactivities of 17 dextrans to (1→3)-specific antibodies to be related to the proportion of (1→3)-linked D-glucopyranosyl residues in each dextran.⁹⁸ *Leuconostoc mesenteroides* NRRL B-1355S and NRRL B-1501S and *Streptococcus viridans* NRRL F90A dextrans precipitated the most antibody nitrogen and, in consequence, many chains in these dextrans would appear to be terminated by nigerosyl groups, as in 45. A dextran (NRRL B-1142) that contains a high percentage of (1→3)-D-glucopyranosidic linkages²¹ had a strikingly low reactivity with this antidextran. To explain this finding, it was suggested^{98,176} that few of the branch chains are terminated by nigerosyl groups (as shown in structure 45).

(178) P. Z. Allen, *Methods Carbohyd. Chem.*, **5**, 232 (1965).

Of 17 dextrans tested with a (1→2)-specific antidextran, *Leuconostoc mesenteroides* NRRL B-1299S, NRRL B-1299L, NRRL B-1399, and NRRL B-1424 dextrans had the highest proportions of (1→2)-linked α -glucopyranosyl residues and precipitated the greatest amounts of antibody nitrogen.⁹⁸ It was, therefore, concluded that numerous chains in these dextrans are terminated by kojibiosyl groups (depicted in structure 44). It has now been established⁹² that all of the (1→2)-linkages in NRRL B-1299S and NRRL B-1299L dextrans form branching points; many of the branches in these dextrans must, therefore, consist of 2-*O*- α -D-glucopyranosyl groups (see partial structure 10, p. 387).

Kabat and associates^{178a} demonstrated that the reactivities of four absorbed rabbit antisera having (1→2)-specificities are likewise directed against terminal kojibiosyl groups (44) in the (1→2)-branched *L. mesenteroides* NRRL B-1397 dextran, as kojibiose (2) and trisaccharides 7 and 8 were effective inhibitors of the immune reaction. This finding, and the results of a catalytic-oxidation experiment (see p. 410), prompted the authors to conclude^{105,120} that a substantial proportion of the branches in NRRL B-1397 dextran consist of 2-*O*- α -D-glucopyranosyl groups (10).

B. Dextran-Antipneumococcal Cross-reactions. Sugg and Hehre¹⁷⁹ first demonstrated that dextrans derived from sucrose cultures of *L. mesenteroides* strains are able to cross-react with rabbit antibodies to the capsular polysaccharides of Types II and XX (and, sometimes, Type XII) pneumococci. Dextrans cross-reacting only weakly with Type XII pneumococcal antibodies were arbitrarily termed serotype A dextrans. Dextrans cross-reacting strongly with Types II, XX, and XII pneumococcal antibodies were termed serotype B dextrans. Further studies^{180,181} established that the reactivities of serotype A dextrans are dependent upon the presence of (1→6)-linked α -D-glucopyranosyl residues in the dextran molecules, whereas the characteristic reactivities of serotype B dextrans are due to the presence of additional, (1→2)-linked α -D-glucopyranosyl residues.¹⁰³

Goodman and Kabat¹⁸² examined the cross-reactions of 23 dextrans with both horse and rabbit antibodies to Types II and XX pneumococci. All of the dextrans tested precipitated considerable quantities of antibody nitrogen, the most being generally precipitated by the dextrans having the highest proportions of (1→6)-glucopyranosidic linkages. Quantitative inhibition-studies with oligosaccharides provided the specificities of

(178a) M. Torii, K. Sakakibara, and E. A. Kabat, *J. Immunol.*, **110**, 951 (1973).

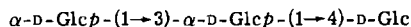
(179) J. Y. Sugg and E. J. Hehre, *J. Immunol.*, **43**, 119 (1943).

(180) E. J. Hehre, *Abstr. Papers Amer. Chem. Soc. Meeting*, **129**, 3D (1956).

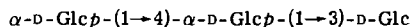
(181) M. Heidelberger and P. A. Rebers, *J. Bacteriol.*, **80**, 145 (1960).

(182) J. W. Goodman and E. A. Kabat, *J. Immunol.*, **84**, 333 (1960).

the cross-reactions;¹⁸² the results indicated that terminal, nonreducing sequences (branch chains) composed of at least four (1→6)-linked α-D-glucopyranosyl residues (structure 36; $n = 2$; see p. 396) are present in all 23 of the dextrans, although such sequences appear to be fewest in number in certain highly branched dextrans. The abilities of the same group of dextrans to precipitate horse antibodies to Types IX and XII pneumococci were found to be related to the content of secondary D-glucopyranosidic linkages in each dextran.⁹⁹ Oligosaccharide inhibition-studies provided evidence that these cross-reactions involve populations of antibodies having heterogeneous specificities; this circumstance limited the structural information that might otherwise have been gained from their study. *Leuconostoc mesenteroides* NRRL B-1355S, NRRL B-1498S, and NRRL B-1501S dextrans [which contain high percentages of (1→3)-linked D-glucopyranosyl residues²¹] precipitated the most Type IX antibody nitrogen. Nigerose (3, see p. 378) was the most effective inhibitor of the cross-reaction,^{99,178} indicating that many chains in these dextrans are terminated by nigerosyl groups (shown in structure 45, p. 400). *L. mesenteroides* NRRL B-1299S and NRRL B-1299L dextrans contain fewer (1→3)-linked residues (see Table VI, p. 384), but are almost as effective in precipitating the Type IX antibodies. Nigerose (3) and, especially, the trisaccharides *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose (49) and *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-D-glucose (50), derived from the polysac-

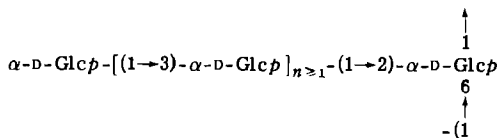


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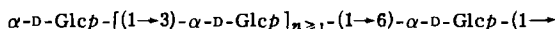


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charide nigeran, inhibited the cross-reaction. As a result, it seems probable that the (1→3)-α-D-glucopyranosidic linkages in these dextrans are mainly contained within terminal, nonreducing sequences of the kind represented by partial structures 51 or 52, or both. *Leuconostoc mesenteroides* NRRL B-1299S, NRRL B-1299L, NRRL B-1399, and NRRL



51



52

B-1424 dextrans [which possess the highest proportions of (1→2)-D-glucopyranosidic linkages among the dextrans tested] gave the strongest precipitin-reaction with Type XII pneumococcal antibodies.⁹⁹ The cross-reaction was effectively inhibited by kojibiose (2, see p. 378), from which it was concluded^{99,176} that chains in these dextrans are terminated by kojibiosyl groups (44, p. 400). *Leuconostoc mesenteroides* NRRL B-742S, NRRL B-1355S, NRRL B-1498S, and NRRL B-1501S dextrans [which contain few, if any, (1→2)-linked D-glucopyranosyl residues^{21,111}] were also able to precipitate considerable amounts of the Type XII pneumococcal antibodies. Two trisaccharides (49 and 50) derived from nigeran were the most effective inhibitors of the cross-reactions, indicating that the specificity is directed against terminal, nonreducing sequences in these dextrans that include (1→3)- or (1→4)-linked α -D-glucopyranosyl residues, or both.

D-Glucopyranosyl residues linked α -(1→6), α -(1→3), and α -(1→2) have been found to be involved in the cross-reactions between dextrans and a horse Type XXII antipneumococcal serum.^{182a} Amongst the dextrans tested, *L. mesenteroides* NRRL B-1299S, NRRL B-1299L, NRRL B-1355S, NRRL B-1399, and NRRL B-1501S dextrans, which contain high percentages of (1→2)- and/or (1→3)-linked residues,²¹ precipitated the greatest amounts of antibody nitrogen, a finding that suggests that a large proportion of the branch chains in these dextrans are terminated by kojibiosyl (44) and/or nigerosyl (45) groups. The cross-reaction between NRRL B-1299S dextran and the Type XXII antiserum (absorbed with NRRL B-512 dextran) was subsequently re-investigated by Kabat and associates.^{178a} On a molar basis, nigerotriose (15), nigerotetraose (37, $n = 2$), nigeropentaoose (37, $n = 3$), and trisaccharides 7 and 8 proved to be the best inhibitors of the immune reaction, suggesting that some branch chains in this (1→2)-branched dextran (see Table VI, p. 384) consist of 2-O- α -D-glucopyranosyl groups (10), whereas others are terminated by α -nigero-oligosaccharidyl groups (structure 51 or 52, or both).

C. Dextran-Antiteichoic Acid Cross-reactions. Knox and Wicken¹⁸³ demonstrated that *L. mesenteroides* NRRL B-512, NRRL B-742, NRRL B-1355, and NRRL B-1415 dextrans cross-react strongly when incubated with antisera to *Lactobacillus planarum* NCIB 7220 ribitol teichoic acid or glycerol teichoic acid. Hapten inhibition-studies, together with the finding that these antibodies react against substituent D-glucopyranosyl groups on the ribitol or glycerol residues of the teichoic acids, prompted

(182a) J. W. Goodman and E. A. Kabat, *J. Immunol.*, **85**, 213 (1964).

(183) K. W. Knox and A. J. Wicken, *Arch. Oral Biol.*, **17**, 1491 (1972).

the conclusion that these cross-reactions are mediated through numerous, branched segments in the dextran molecules in which the branch chains are composed of single α -D-glucopyranosyl groups.

(v) **Dextran-Concanavalin A Interaction.** Initial investigations of the precipitin reactions that occur between native dextrans and concanavalin A, a globulin from *Canavalia ensiformis*, were undertaken by F. Smith and associates¹⁸⁴ (see also, Refs. 103 and 185). A close correlation was discovered between the ability of dextrans to precipitate concanavalin A and Type XII pneumococcal antibodies (see p. 403) that led to a suggestion that (1 \rightarrow 2)-linked α -D-glucopyranosyl residues might be involved in the concanavalin A-dextran interaction.¹⁸⁵ Goldstein and coworkers¹⁸⁶⁻¹⁸⁸ demonstrated that a large number of dextrans precipitate purified preparations of concanavalin A, and provided evidence¹⁸⁹ that concanavalin A has combining sites that are complementary to an α -D-glucopyranosyl unit having free hydroxyl groups at C-3, C-4, and C-6. Thus, the ability of most native dextrans to precipitate concanavalin A would appear to be determined primarily by the number of terminal, nonreducing α -D-glucopyranosyl groups (and, hence the number of branch chains) in the dextran molecule. Preobrazhenskaya and Rozenfel'd²² observed that the ability of dextrans to precipitate concanavalin A is greatly lessened when the lengths of the branch chains are shortened by enzymic hydrolysis, and they proposed that some factor additional to the degree of branching might affect the ability of a dextran to precipitate concanavalin A. The inability of insolubilized concanavalin A to interact with several highly branched dextrans,¹⁹⁰ together with the fact that dextrans are able to precipitate structurally diverse proteins from solution,¹⁹¹⁻¹⁹³ suggest that this factor may relate to the (non-specific) ability of a dextran network (see p. 413) to exclude molecules of concanavalin A from the volume of solvent it circumscribes in solution (see Refs. 191-193).

The nature of the concanavalin A-dextran interaction has permitted

- (184) J. A. Cifonelli and F. Smith, *J. Amer. Chem. Soc.*, **79**, 5055 (1957).
- (185) E. J. Hehre, *Bull. Soc. Chim. Biol.*, **42**, 1581 (1960).
- (186) I. J. Goldstein, *Biochim. Biophys. Acta*, **97**, 68 (1965).
- (187) I. J. Goldstein and L. L. So, *Arch. Biochem. Biophys.*, **111**, 407 (1965).
- (188) I. J. Goldstein, C. E. Hollerman, and J. M. Merrick, *Biochim. Biophys. Acta*, **97**, 68 (1965).
- (189) I. J. Goldstein, C. E. Hollerman, and E. E. Smith, *Biochemistry*, **4**, 876 (1965).
- (190) E. H. Donnelly and I. J. Goldstein, *Biochem. J.*, **118**, 679 (1970).
- (191) T. C. Laurent, *Biochem. J.*, **89**, 253 (1963).
- (192) T. C. Laurent, *Acta Chem. Scand.*, **17**, 2664 (1963).
- (193) T. C. Laurent and J. Killander, *J. Chromatogr.*, **14**, 317 (1964).

concanavalin A to be used as a reagent for studying dextran structures. The finding^{109,186,188} that purified concanavalin A is precipitated by dextrans containing as few as 5% of branch-linkages, but not by unbranched polysaccharides, makes concanavalin A a particularly sensitive reagent for rapidly determining whether a dextran has a linear or a branched structure. Moreover, the degree of branching of a dextran may be estimated approximately by comparing the amounts of concanavalin A precipitated by the dextran studied and dextrans having known degrees of branching. A large number of native dextrans have been tested with concanavalin A, and, without exception, they precipitate the reagent.¹⁸⁶⁻¹⁸⁸ A conclusion implicit from this observation is that all enzymically synthesized, native dextrans have branched structures. A comparison^{109,188} of the reactivity, with concanavalin A, of *Leuconostoc mesenteroides* NRRL B-1375 and NRRL B-1355S dextrans afforded evidence of unusual structural segments in the latter. The NRRL B-1375 dextran, which contains 20% of (1→3)-branch linkages (see Table VI, p. 384), was able to precipitate concanavalin A almost as effectively as NRRL B-1355S dextran [which contains 47% of (1→3)-linkages (see Tables II, p. 379, and V, p. 383)]. This finding suggested that the greater proportion of the (1→3)-linkages in NRRL B-1355S dextran is present in linear segments of the molecule. Similar structural segments may also occur in the dextrans synthesized by *L. mesenteroides* strains 44-2, SF-4, and NRRL B-1389 and *Streptococcus viridans* NRRL B-1351; these give weak precipitates^{22,188} with concanavalin A, and yet contain high percentages of secondary α -D-glucopyranosidic linkages.^{21,22}

(vi) **The Lengths of the Branches in Dextrans: Catalytic Oxidation and *p*-Toluenesulfonylation Experiments.** Indications of the lengths of the branch chains in native dextrans stemmed initially from the results of statistical analysis of fragments of low molecular weight obtained from acid hydrolyzates of dextrans,⁹⁷ and from calculations based upon physicochemical measurements of dextran solutions.¹⁹⁴⁻¹⁹⁷ Additional information has since been obtained from studies of immunochemical reactions and from characterization of the products in enzymic hydrolyzates and acetolyzates of dextrans. The results of some of these experiments are summarized in Table VII.

More-specific information concerning the lengths of the branches in

- (194) M. Wales, P. A. Marshall, and S. G. Weissberg, *J. Polym. Sci.*, **10**, 229 (1953).
- (195) F. R. Senti, N. N. Hellman, N. H. Ludwig, G. E. Babcock, R. Tobin, C. A. Glass, and B. L. Lamberts, *J. Polym. Sci.*, **17**, 527 (1955).
- (196) (a) F. A. Bovey, *J. Polym. Sci.*, **35**, 167 (1959); (b) **35**, 183 (1959).
- (197) E. Antonini, L. Bellelli, M. R. Bruzzesi, A. Caputo, E. Chiacone, and A. Rossi-Fanelli, *Biopolymers*, **2**, 27 (1964).

TABLE VII
Lengths of the Branches in Some Dextrans

Dextran	Number of α -D-glucopyranosyl residues in the branch chain					
	1	$\geq 2-3$	$\geq 4-5$	≥ 6	≥ 8	≥ 50
<i>L. mesenteroides</i>						
NRRL B-512	+ ^{a-d}	+ ^{d,e}	+ ^{a,f}		+ ^f	+ ^g
B-742S	+ ^{a,b}	+ ^f	+ ^a			
B-742L	+ ^b		+ ^a			
B-1298	+ ^h					
B-1299S	+ ^{a,b}	+ ^h	+ ^{a,e}			
B-1299L	+ ^a		+ ^a			
B-1355S	+ ^a		+ ^{a,b}			
B-1375	+ ^{c,e}		+ ^a	+ ⁱ		
B-1397	+ ^{a,c}	+ ^h				
B-1399			+ ^a			
B-1415	+ ^{a,c,e,i}	+ ^f				
B-1416	+ ⁱ					
<i>S. viridans</i> var.						
NRRL F90A			+ ^a			
<i>Streptobacterium dextranicum</i>						
NRRL B-1255			+ ^a			

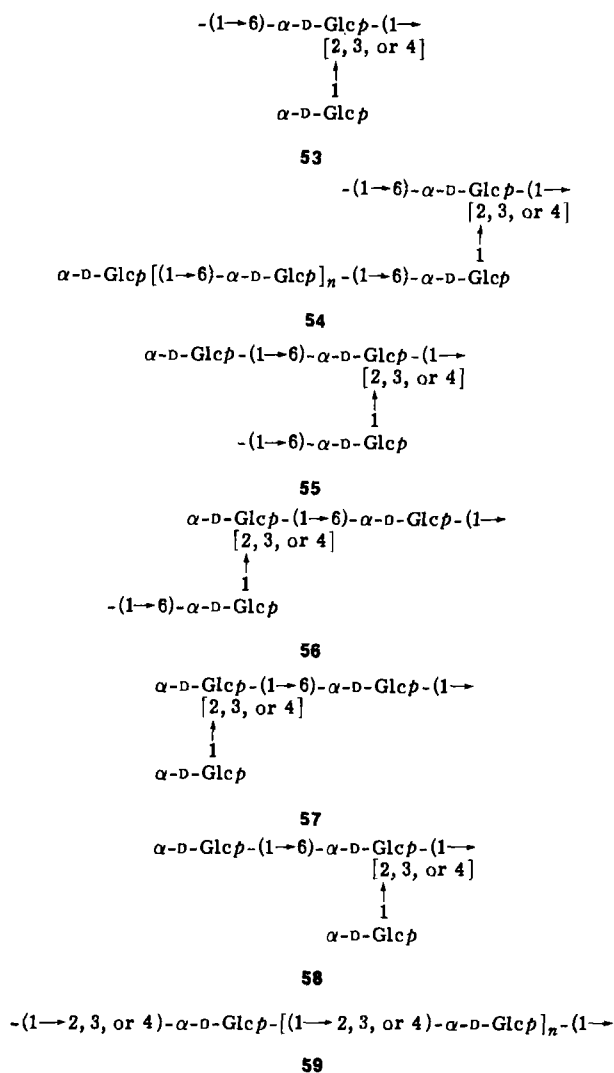
^a Immunochemistry (see p. 399). ^b Acid hydrolysis.⁹⁷ ^c Catalytic oxidation (see p. 407). ^d Lindberg degradation (see p. 411). ^e Dextranase (see p. 391). ^f 6-Glucanohydrolase (see p. 396). ^g Physicochemical measurements.¹⁹⁴⁻¹⁹⁷ ^h Acetolysis (see p. 386). ⁱ Dextranoglucosidase (see p. 395). ^j Glucoamylase (see p. 398).

several dextrans has now been obtained by examination of fragments from dextrans that had then been catalytically oxidized or *p*-toluene-sulfonylated, respectively.

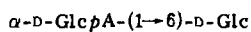
The fact that the rate of acid hydrolysis of methyl α -D-glucopyranoside is greatly lessened when the hydroxymethyl group on C-5 is replaced by a carboxyl group^{198,199} prompted¹⁰⁰ the suggestion that it might be possible to stabilize structural segments in a dextran with respect to acid hydrolysis by catalytically oxidizing the hydroxymethyl groups in the dextran to carboxyl groups, and then identify them by characterizing the acidic oligosaccharides obtained on hydrolyzing the oxidized dextran with acid. Some segments that may be present in dextrans, and that could be stabilized by catalytic oxidation, are depicted in partial structures 53 to 59. On catalytic oxidation and acid hydrolysis, a characteristic,

(198) D. B. Easty, *J. Org. Chem.*, **27**, 2102 (1962).

(199) B. Capon and B. C. Ghosh, *Chem. Commun.*, 586 (1965).



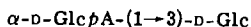
secondary-linked aldobiouronic acid would be obtained from structure **53**, and isomaltobiouronic acid (**60**) from structures **54**, **55**, and **56**. Structures **57** and **58** would yield characteristic aldotriouronic acids con-



Isomaltobiouronic acid

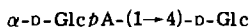
60

taining two carboxyl groups, and structure 59, linear aldouronic acids having structures dependent upon the lengths of the sequences of secondary-linked D-glucopyranosyl residues in the segment. The structures of a number of native^{100,120,125} and acid-degraded¹²⁵ dextrans have been examined by this degradative procedure. Nigerobiouronic acid (61) and maltobiouronic acid (62) were, respectively, the only acidic oligosac-



Nigerobiouronic acid

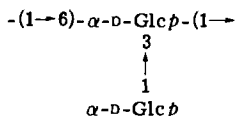
61



Maltobiouronic acid

62

charides identified in acid hydrolyzates of catalytically oxidized *Leuconostoc mesenteroides* NRRL B-1375 and NRRL B-1415 dextrans.¹⁰⁰ As all of the secondary linkages in these dextrans form branch points (see Table VI, p. 384), these disaccharidic acids must have come from oxidized segments of the kind represented by structure 53. The yield of nigerobiouronic acid (61) from NRRL B-1375 dextran, moreover, established that at least 62% of the branches are 3-O- α -D-glucopyranosyl groups (as in partial structure 63); and the proportion of maltobiouronic acid

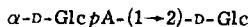


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(62) from NRRL B-1415 dextran indicated that at least 50% of the branches in the dextran are 4-O- α -D-glucopyranosyl groups (as in partial structure 43; see p. 399). The failure to isolate isomaltobiouronic acid (60) from either of the hydrolyzates suggested that these dextrans contain few segments of the kind represented by structures 54 and 55. Segments of this type are, however, present in the (1 \rightarrow 3)-branched NRRL B-512 dextran (see Table VI, p. 384), as it furnished isomaltobiouronic acid (60) and nigerobiouronic acid (61) upon catalytic oxidation and acid hydrolysis.¹²⁵ The ratio of the amounts of the two acids (4:1) and the degree of oxidation of the dextran (60%) indicated that at least 48% of the branches either consist of (a) two or more (1 \rightarrow 6)-linked α -D-glucopyranosyl residues (structure 54), or (b) 6-O- α -D-glucopyranosyl groups

(structure 55), and that a minimum of 12% of the branches are 3-*O*- α -D-glucopyranosyl groups (structure 63).

Following catalytic oxidation and acid hydrolysis, the (1 \rightarrow 2)-branched *L. mesenteroides* NRRL B-1397 dextran (see Table VI) yielded kojibouronic (63a) and isomaltobouronic (60) acids, together with several



63a

higher, acidic oligosaccharides.¹²⁰ The combined yields of fragments that contained kojibouronic acid residues provided evidence that a minimum of 19% of the branches in the native dextran are 2-*O*- α -D-glucopyranosyl groups (10, p. 387).

The increased stability towards acid conferred on an α -D-glucopyranosidic linkage by oxidation of the hydroxymethyl group on C-5 of the D-glucopyranosyl group to a carboxyl group has been ascribed to the greater electronegativity of the carboxyl group.¹⁰⁹ Hirst and coworkers¹¹⁴ observed that the *p*-tolylsulfonyloxymethyl group exerts an inductive effect similar to that of the carboxyl group, and proposed that structural segments in a dextran might be stabilized with respect to acid hydrolysis by replacing the hydroxymethyl groups by *p*-tolylsulfonyloxymethyl groups, and then be identified by characterizing the *O*-(*p*-toluenesulfonylated) oligosaccharides obtained on acid hydrolysis of the *O*-(*p*-toluenesulfonylated) dextran.

This degradative scheme was employed in investigating the structure of *Leuconostoc mesenteroides* NCIB 2706 dextran.¹¹⁴ Selective *O*-(*p*-toluenesulfonylation) of the primary hydroxyl groups in the dextran was achieved after replacing the secondary hydroxyl groups by methoxyl groups: this involved successive tritylation, methylation, and detritylation of the dextran. On acid hydrolysis, the resulting *O*-methyl-*O*-*p*-tolylsulfonyl derivative of the dextran gave *O*-methyl-*O*-*p*-tolylsulfonyl derivatives of isomaltose (1) and nigerose (3) in the ratio of 13 to 1. As almost all of the secondary linkages form branch points in the dextran (see Table VI, p. 384), these fragments established that the native dextran contains segments represented by partial structures 54, 55, and 63, or 54 or 55 and 63. A detailed analysis of the resulting disaccharide derivatives confirmed that the majority of the branches are of the kind represented by structure 54 (see p. 408).

A limitation on the experimental methods discussed in the previous paragraphs arises from the fact that they permit determination of only the percentages (not the precise lengths) of the branches that are terminated by 6-*O*- α -D-glucopyranosyl groups. This limitation was

partially overcome by Lindberg and associates,^{101,125} who devised a scheme for studying branch lengths that involves sequentially degrading the branch chains by removing single D-glucopyranosyl groups from the chain ends (so eliminating branches composed of a single α -D-glucopyranosyl group) and measuring the lessening in the degree of branching of the dextran that results after each degradational step.

This scheme of branch analysis was employed¹⁰¹ to re-examine the lengths of the branch chains in *Leuconostoc mesenteroides* NRRL B-512 dextran. Selective removal of D-glucopyranosyl groups was accomplished by (a) replacing the hydroxymethyl groups in the dextran by C-(*p*-tolylsulfonyl)methyl groups, and (b) treating the modified dextran with alkali.²⁰⁰ Lessening of the degree of branching was calculated from methylation or ethylation analyses of the dextran before and after each sequential degradation. The first degradation eliminated 40% of the branch chains, and so, these must consist of D-glucopyranosyl groups (see structure 63). A second degradation removed an additional 45% of the branches; therefore, these are composed of two D-glucopyranosyl residues (structure 54; $n = 0$). The remaining 15% of the branches necessarily consist of three or more residues (structure 54; $n > 1$). These findings compared favorably with those obtained from a catalytic oxidation experiment (see p. 409), but may be contrasted with conclusions, derived from a statistical analysis of fragments from an acid hydrolyzate of NRRL B-512 dextran,⁹⁷ that indicated that 77% of the branches consist of single α -D-glucopyranosyl groups. In the latter analysis, however, no distinction was possible between branches composed of a 3-*O*- α -D-glucopyranosyl group (see structure 63) and a 6-*O*- α -D-glucopyranosyl group (structure 55, p. 408).

c. Conclusions.—The main features of the chemical structures of the dextrans that have been considered in this Section may be summarized as follows.

(i) **Skeletal Chain Structures.** Dextran chains are composed of sequences of (1→6)-linked α -D-glucopyranosyl residues, some of which carry branches at either C-2, C-3, or C-4. Isolated, (1→3)-linked, α -D-glucopyranosyl residues, or sequences of these residues, may intersect or terminate the skeletal chains of some dextrans.

(ii) **Secondary Linkages.** All of the native dextrans that have been examined to date contain secondary α -D-glucopyranosidic linkages. Considerable differences exist in the types and proportions of secondary linkages present in dextrans elaborated by different micro-organisms

(linkages that make each dextran characteristic of the particular micro-organism that synthesizes it).

Rather surprisingly, only three different combinations of α -D-linkages are to be regularly found in dextran preparations: they all contain (1 \rightarrow 6) and (1 \rightarrow 3) linkages, and some possess additional (1 \rightarrow 2) or (1 \rightarrow 4) linkages. It is not at present possible to assess the reasons for these distributions of secondary linkages; they could, for instance, merely reflect the fact that certain micro-organisms concurrently synthesize two or more polysaccharides, although, should this occur, it is somewhat surprising that (1 \rightarrow 6) linkages have not been found in combination with only (1 \rightarrow 2) or (1 \rightarrow 4) linkages (or both) in some dextran preparations. On the other hand, the distribution may be occasioned by an inherent property of dextran-synthesizing enzymes to direct transferred α -D-glucopyranosyl groups to the secondary hydroxyl groups on C-2 and C-3, or C-3 and C-4, of a growing dextran molecule. With the exception of (1 \rightarrow 3) linkages, secondary linkages form branch points in native dextrans.

(iii) **Branching.** Dextran preparations that have been examined by methylation analysis (see Table VI, p. 384) contain 5 to 33% of branching D-glucopyranosyl residues. These branching-residues appear to be distributed randomly throughout the dextran molecule, and most (if not all) of them carry only a single, secondary branch-linkage at C-2, C-3, or C-4. Most of the branches in the dextrans that have been examined to date consist of a single α -D-glucopyranosyl group, although evidence has been presented that indicates that branches composed of between 2 and 50 (1 \rightarrow 6)-linked residues exist in some dextrans (see Table VII, p. 407). Furthermore, it seems probable that the branch chains in a few dextrans are formed of sequences of (1 \rightarrow 3)-linked residues.

(iv) **Segment and Type Structures.** Linear structures **6** (see p. 386) and **36** (see p. 396), and the branched structures **53** and **54**, are segments common to most of the dextrans discussed in this Section. The branched structure **55** has also been identified in a single dextran. Partial structures **26** (p. 390) or **38** (or both) form major segments in two water-insoluble dextrans and, as water-insoluble dextrans usually contain high percentages of (1 \rightarrow 3)-linked residues,^{21,92,126} they could conceivably be segments common to this group of dextrans. At present, the structure of no dextran has been studied in sufficient detail to permit an unequivocal assignment of a type structure to the molecule. The partial structures that have been identified in dextrans could, for instance, represent segments of molecules having comb-like, laminated, or ramified types of structures (see Fig. 2). Dextrans are, nevertheless, generally assumed to have ramified structures. In support of this view, it has been

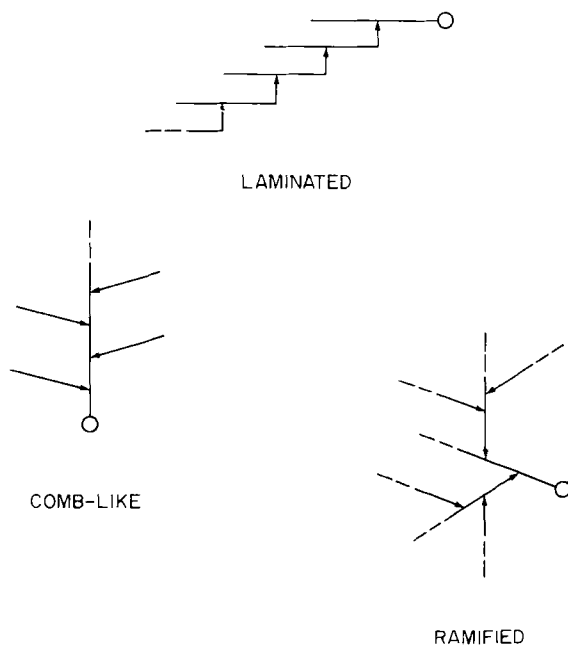


FIG. 2.—Schematic Representations of Possible Types of Dextran Structures. {For clarity, short branch-chains are omitted. \bigcirc = terminal, reducing-end residue; \rightarrow = secondary α -linkages; — = chains of (1 \rightarrow 6)-linked [sometimes (1 \rightarrow 3)-linked] α -D-glucopyranosyl residues.}

suggested,²⁰¹ moreover, that a polymer having this type of structure would have light-scattering properties similar to those exhibited by dextran molecules in dilute solution.

2. Network Structures

To facilitate the examination of the network structures that are formed by dextrans, the salient features of polysaccharide networks will initially be summarized.

In the presence of aqueous solvents, polysaccharides typically form three-dimensional network structures known as gels.^{202,203} In these networks may be recognized amorphous regions composed of (unassociated)

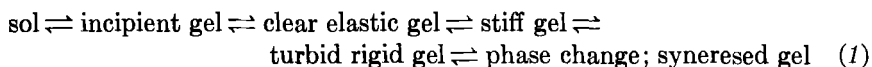
(201) G. Y. Beke and T. Gilanyi, *Ann. Univ. Sci. Budapest Rolando Eotvos Nominatae, Sect. Chim.*, **12**, 95 (1971).

(202) D. A. Rees, *Advan. Carbohydr. Chem. Biochem.*, **24**, 267 (1969).

(203) (a) R. L. Miller, in "Encyclopedia of Polymer Science and Technology," J. Conrad, ed., John Wiley and Sons, Inc., New York, 1966, Vol. 4, p. 449.
(b) R. H. Marchessault and A. Sarko, *Advan. Carbohydr. Chem.*, **22**, 421 (1967).

polysaccharide chain segments in the random-coil conformation, and crystalline regions (termed crystallites, micelles, or junction zones) in which associated chain-segments have a regularly ordered stereochemistry. The differences between polysaccharide gels appear to be determined largely by the nature and number of the intermolecular or cross linkages between chain segments.^{202,203} These variables are, in turn, dependent upon the chemical structures of the polysaccharides, and their interactions with the solvent system that enable the chain segments to attain characteristic conformations at the junction zones.

Rees²⁰⁴ distinguished a series of stages leading to the formation of different types of polysaccharide gel (equilibrium 1)



In the sol state, polysaccharide chains usually assume the random-coil conformation, but this is converted into a flexible gel when sufficient cross-linkages to provide a continuous network have formed. The formation of further cross-linkages imparts greater rigidity to the gel, and optical transparency may eventually be lost. A syneresed gel is produced when the tendency to form cross-linkages is so strong that the network contracts, with consequent exclusion of some solvent molecules.

As indicated earlier in the present Section, dextrans vary greatly in chemical structure. These differences are reflected in the types of gels formed by different dextrans; the gel may have properties approaching those of a sol or a syneresed gel.

a. Gels Formed by Water-soluble Dextrans.—In the presence of water, dextrans of this group form, at high concentrations of the dextran, tacky pastes that are readily dispersed at room temperature on dilution with further amounts of solvent.²¹ The development of crystallinity in the pastes formed by allowing freeze-dried dextrans to absorb water at room temperature,²⁰⁵⁻²⁰⁷ and the observation that films formed by evaporation of dilute solutions of dextrans are composed of networks of microscopic filaments,^{81,208,209} together provide evidence that dextrans of

(204) D. A. Rees, *Chem. Ind.* (London), 630 (1972).

(205) A. (R.) Jeanes, N. C. Schieltz, and C. A. Wilham, *J. Biol. Chem.*, **176**, 617 (1948).

(206) N. W. Taylor, H. F. Zobel, N. N. Hellman, and F. R. Senti, *J. Phys. Chem.*, **63**, 599 (1959).

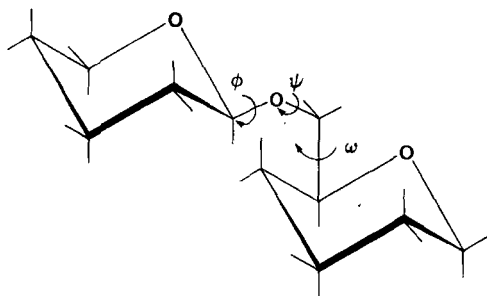
(207) N. W. Taylor, J. E. Cluskey, and F. R. Senti, *J. Phys. Chem.*, **65**, 1810 (1961).

(208) B. Ingelman and K. Siegbagn, *Nature*, **154**, 237 (1944).

(209) P. Critchley and C. A. Saxton, *Abstr. Papers Int. Ass. Dent. Res.* (Brit. Div.), **19**, 45 (1971); *J. Dent. Res.*, **50**, 1175 (1971).

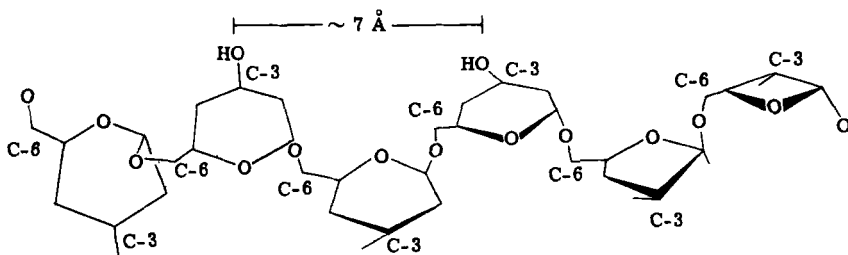
this group form gel structures. Molecular-weight determinations,⁸⁰ electron-microscopic observations,⁸¹ and the nature of the interactions of dextrans with several proteins,¹⁹¹⁻¹⁹³ moreover, suggest that many of these dextrans remain associated, presumably in some form of network structure, even in dilute solution. It appears likely^{206,207} that, in the most-ordered gels, formed by water-soluble dextrans, the percentage of crystalline material does not exceed 30% of the total and must be substantially less in most cases. Such low crystallinity favors swelling and dissolution; hence, the gels formed by this group of dextrans are characterized by their flexibility and ease of dispersal.

The skeletal chains of most water-soluble dextrans appear to be composed of sequences of (1→6)-linked α -D-glucopyranosyl residues to which branches that often consist of a single α -D-glucopyranosyl group are attached at irregular intervals. For present purposes, this group of dextrans may, therefore, be visualized as being composed of a continuous series of branched and linear regions. Comparisons of the X-ray diffraction powder diagrams of retrograded-native and acid-hydrolyzed dextrans,²⁰⁵⁻²⁰⁷ and of an essentially unbranched, chemically synthesized dextran⁴ indicated that, in general, the water-soluble dextrans that crystallize best contain the highest proportions of (1→6)-linked residues. This fact, and the similarities in the X-ray powder diagrams given by several different water-soluble dextrans, suggest that dextrans of this group form network structures as a result of association of linear chain segments of the kind represented by structures **6** (see p. 386) and **36** (see p. 396). *Leuconostoc mesenteroides* NRRL B-1355S dextran²⁰⁶ is an exception to this rule, and it may form networks by association of other linear segments (see structure **18**, p. 388). Insufficient X-ray data are, as yet, available to permit identification of the conformation (or conformations) of the chain segments (and, hence, the arrangements of these chains) at the junction zones of the gels formed by this group of dextrans. Some speculations as to the nature of the chain conformations are, nevertheless, possible. Measurements of p.m.r. spectra indicate that the individual D-glucopyranosyl residues in dextrans are in the 4C_1 conformation.¹¹³ Thus, if a fixed value is assumed for the bond angle at the glycosidic oxygen atom, only three variables remain to be defined in order to specify the geometry of the linear chain segments depicted in structures **6** and **36**: these are the rotational angles ϕ , ψ , and ω shown in formula **64**. It may be assumed that these rotational angles will have the same set of values at all of the (1→6) linkages within the chain segments forming the junction zones, as these are ordered regions of the gel network. In consequence, these chains are most likely to have regular (although not necessarily integral), helical conformations.²¹⁰ Computer model-building



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procedures have been employed²¹⁰ to determine the numbers of minimum-energy ("allowed"), helical conformations that a regular chain of (1→6)-linked α -D-glucopyranosyl residues may assume when ϕ , ψ , and ω are altered independently in steps of 10° . The results of these experiments indicated that the number of allowed conformations is very large, with numbers (n) of residues per helix turn ranging from ± 7 to ± 2 , and projected lengths (h) of residues along the helix axis ranging from 0 to 6 Å. The distribution of n and h values, however, suggested a bias towards extended (ribbon-like) conformations in which $n = 2$ to ± 3 and $h = 4$ to 6 Å. A representative, extended conformation (after Ebert²¹¹) having 2 residues per helix turn is shown in formula 65. As-



65

sociation of chain segments of dextrans held in this conformation would be possible through intermolecular hydrogen-bonds between regularly spaced 3-hydroxyl groups.

(210) (a) D. A. Rees, and W. E. Scott, *J. Chem. Soc. (B)*, 469 (1971); (b) D. A. Rees, in "MTP International Review of Science," D. H. Hey and G. O. Aspinall, eds., Butterworth, London, 1973, Vol. 7, p. 251.

(211) K. H. Ebert, *Monatsh.*, **98**, 1128 (1967).

b. Gels formed by Water-insoluble Dextrans.—D-Glucans of this group form, in water, compacted gels that cannot be dispersed by heating the gel in an excess of the solvent. However, the gels disrupt in the presence of aqueous alkali,²¹ or such dipolar, aprotic solvents as *N,N*-dimethylformamide or methyl sulfoxide,^{21,212} presumably as a consequence of conformational changes caused by interactions of electrostatic charges that result from the ionization of hydroxyl groups in the dextran molecules.²¹³

The chemical structures of only a few water-insoluble dextran preparations have been examined in detail. The results of available structural analyses^{21,92,123a,126} suggest, however, that dextrans of this group either have structures more complex than those of most of the water-soluble dextrans, or that the preparations consisted of mixtures of water-soluble dextrans and unrelated D-glucans. In addition to the structural features found in water-soluble dextrans, preparations of water-insoluble dextrans usually contain high proportions of (1→3)-linked α -D-glucopyranosyl residues.^{21,92,123a,126} In four preparations examined to date, most of the (1→3) linkages were not engaged in branch points,^{92,123a,126,136} and, for two preparations, moreover, it was demonstrated that the (1→3)-linked residues are joined in continuous sequences.^{123,136} The latter finding further suggested that water-insoluble dextrans may generally contain two distinct linear regions [represented by structures 6 (p. 386) and 36 (p. 396) and 26 (p. 393) and 38 (p. 398), respectively], in addition to branched regions.*

Electron-microscope observations³⁵ and chemical analyses^{126,136} of *Streptococcus mutans* OMZ 176 dextran indicated that the gel formed by this water-insoluble dextran is composed of two distinct networks, one formed by association of (1→3)-linked chain segments [structures 26 (p. 393) and 38 (p. 398)], and the other by association of (1→6)-linked chain segments [structures 6 (p. 386) and 36 (p. 396)]. It remains to be determined whether this and similar gels^{21,92,126,212} are formed from a single polysaccharide type (and are, therefore, continuous), or from at least two different polysaccharides.

(212) D. S. Genghof and E. J. Hehre, *Proc. Soc. Exp. Biol. Med.*, **140**, 1298 (1972).

(213) (a) V. S. R. Rao and J. F. Foster, *Biopolymers*, **1**, 527 (1963). (b) K. Ogawa, T. Watanabe, J. Tsurugi, and S. Ono, *Carbohydr. Res.*, **23**, 399 (1972).

* A small number of dextrans that contain few, if any, (1→3)-linked α -D-glucopyranosyl residues also form compacted gels.^{27,110} In these preparations, an unusual distribution of branching residues, or the presence of ionizable groups in the dextran molecules (see Ref. 310), or the presence of peptide molecules as impurities in the preparations (see Ref. 310), may greatly facilitate cross-linking of (1→6)-linked chain segments in the polysaccharides.

The latter possibility is favored by Guggenheim and coworkers,^{123,136,214} who proposed that the gels formed by the group of dextrans (α -glucans) under consideration owe their rigidity to a framework composed of associated chain segments of mainly (1 \rightarrow 3)-linked α -glucans, in the interstices of which are situated network structures formed by water-soluble dextrans. In this arrangement, the final properties of the gel would presumably be dependent upon the proportions of these constituent polysaccharides, although the degree of interlinking between the two networks might be an additional, contributory factor.

The parameters needed for specifying the arrangements of the chain segments of the (1 \rightarrow 3)-linked residues at the junction zones of the gel formed by OMZ 176 dextran (and other water-insoluble dextrans) have not yet been determined. Computer model-building procedures, however, indicated²¹⁰ that associated, (1 \rightarrow 3)-linked chain segments in these gels are most likely to be held in extended (ribbon-like) conformations in which n lies in the range of -4 to $+3$, and h is 3 to 5 Å. In general, these chains may be expected to be rigid and to have skeletal properties comparable with those of chains of (1 \rightarrow 4)-linked β -D-glucopyranosyl residues (such as are found in cellulose).

IV. BIOSYNTHESIS

1. The Enzymes

a. The Branching Enzyme.—An early proposal,⁶ based on the results of Bovey¹⁹⁶ and Stacey and associates,^{215,216} assumed that the skeletal chains of dextrans are synthesized by the enzyme dextranucrase, and the secondary (branch) linkages through the agency of a separate, branching enzyme. The branching enzyme was believed to be more thermally stable than dextranucrase,¹⁹⁶ and to be active only in the presence of magnesium ions.²¹⁵ Subsequent workers failed to demonstrate a particular metal-ion requirement for branched-dextran synthesis,^{48,126,127} or to isolate a separate branching-enzyme by means of refined separatory techniques.^{48,217,218} Dextranucrase would, therefore, appear to be re-

(214) B. Guggenheim, B. Regolati, and H. R. Mühlemann, *Caries Res.*, **6**, 289 (1972).

(215) S. A. Barker, E. J. Bourne, A. E. James, W. B. Neely, and M. Stacey, *J. Chem. Soc.*, 2096 (1955).

(216) R. W. Bailey, S. A. Barker, E. J. Bourne, and M. Stacey, *J. Chem. Soc.*, 3530 (1957).

(217) J. Carlsson, E. Newbrun, and B. Krasse, *Arch. Oral Biol.*, **14**, 469 (1969).

(218) K. H. Ebert and G. Schenk, *Advan. Enzymol.*, **30**, 179 (1968).

sponsible for the synthesis both of the skeletal chains and the branch linkages in dextrans. To account for the structural diversity of dextrans from different biological sources, Hehre and Suzuki²¹⁹ postulated the existence of a family of dextransucrases that differ in their relative affinities for the various hydroxyl groups of the D-glucopyranosyl residues of growing dextran chains.

b. Dextransucrase.—Dextransucrase is a member of a general class of transglycosylases, and has been named in the literature as (1→6)- α -D-glucan: D-fructose-2-D-glucosyltransferase (E.C. 2.4.1.5). This specification implies that the enzyme exclusively catalyzes the synthesis of chains of (1→6)-linked α -D-glucopyranosyl residues from those groups in sucrose. For reasons already indicated, this specification may constitute an incomplete description of the enzyme.

(i) **Preparation and Properties of Dextransucrases.** Enzyme preparations described as dextransucrases have been obtained from species of *Lactobacillus*, *Leuconostoc*, and *Streptococcus*.

It has not been conclusively demonstrated that Lactobacilli elaborate dextransucrases, although the evidence at present available suggests that these enzymes may be constitutive to certain species of the genus. Hammond³⁰ indicated that cell-free extracts of *Lactobacillus casei* (32-1+), an oral strain, synthesize from sucrose a polysaccharide serologically similar to dextran. *Lactobacillus* RWM-13, a strain isolated from decomposing vegetable-matter, may produce a structure-bound, temperature-sensitive dextransucrase.³⁴

Leuconostoc species may be satisfactorily cultured on a variety of carbohydrates, but, for the production of dextransucrases, sucrose must be included in the nutrient medium; indeed, it has been suggested²²⁰ that the enzyme-induction process may be triggered by the D-fructofuranosyl group of the sucrose molecule. In consequence of this condition, preparations of dextransucrases from *Leuconostoc* species have contained various proportions of dextran that could not be removed by any fractionation procedures available. The presence of this tenaciously bound dextran, once regarded as an integral part of the dextransucrase molecule,²²¹ has presented a major obstacle to further study of the enzyme. Stacey and associates²²² attempted to produce a dextran-free enzyme by growing *L. mesenteroides* NRRL B-1375 on a sucrose medium contain-

(219) E. J. Hehre and H. Suzuki, *Arch. Biochem. Biophys.*, **113**, 675 (1966).

(220) W. B. Neely and J. Nott, *Biochemistry*, **1**, 1136 (1962).

(221) E. J. Hehre, *Advan. Enzymol.*, **11**, 297 (1951).

(222) R. W. Bailey, S. A. Barker, E. J. Bourne, and M. Stacey, *J. Chem. Soc.*, 3536 (1957).

ing a large excess of maltose (4, see p. 378). The resulting enzyme had, however, only a limited capacity to synthesize dextran from sucrose. Ebert and Schenk²¹⁸ reported some success in preparing, from several strains of *L. mesenteroides*, dextransucrases that contained relatively small proportions of dextran. Storage of the most highly purified enzyme preparations at low temperature produced active precipitates in which most of the enzyme molecules may have been free from dextran (as the precipitates were solubilized by addition of dextran or sucrose). The sedimentation patterns of *L. mesenteroides* NRRL B-512 dextransucrase revealed the presence of a protein of uniform molecular weight, estimated to be of the order of 280,000. Differences were not observed in the synthetic activity of this dextransucrase at various stages of purification, from which it was concluded that the enzyme catalyzes the synthesis both of the skeletal chains and the branch-linkages of NRRL B-512 dextran.

Most of the *Leuconostoc* strains so far characterized synthesize only exocellular, water-soluble dextrans,²¹ and it may be presumed that they therefore secrete exocellular dextransucrases. Members of a small group of *Leuconostoc* micro-organisms, however, elaborate additional dextrans that are insoluble in the bacterial fermentation-mixtures.²¹ The bio-synthetic relationships existing between the polysaccharides soluble in the fermentation mixture and the insoluble polysaccharides synthesized by *L. mesenteroides* NRRL B-1299 have been examined by E. E. Smith.²²³ The rates of incorporation of ¹⁴C-labelled α -D-glucopyranosyl groups into the polysaccharide fractions soluble and insoluble in the medium suggested that at least 60% of the insoluble polysaccharide in the fermentation mixture consisted of a complex between dextransucrases (glucosyltransferases) bound to the bacterial cell-wall and water-soluble dextrans in various stages of synthesis, and that the complex breaks down on completion of the synthetic reaction, to release the water-soluble, native dextran into the supernatant solution. Subsequent examination of purified *L. mesenteroides* NRRL B-1299 dextrans revealed that up to 10% of the native dextran synthesized by the structure-bound enzymes is inherently water-insoluble.⁹² Structural differences between the purified water-soluble and water-insoluble dextrans (see Table VI, p. 384) suggest that the *L. mesenteroides* NRRL B-1299 micro-organism may elaborate more than one structure-bound D-glucosyltransferase.

The principal dextransucrase activity of *Leuconostoc dextranicum* (strain *elei*²²⁴) also appears to be associated with the bacterial structure.

(223) E. E. Smith, *FEBS Lett.*, **12**, 33 (1970).

(224) W. W. Carlson, C. L. Rosano, and V. Whiteside-Carlson, *J. Bacteriol.*, **65**, 136 (1953).

Detailed studies of the enzyme(s) or of the structures of the dextrans produced have not, however, been published.

In general, dextransucrase preparations from *Leuconostoc* species exhibit optimal activity at pH 5–5.5 and 29–34°; they synthesize dextrans of high molecular weight ($\bar{M} \sim 5 \times 10^6$) having 5 to 35% of branch-linkages. In the form of cell-free extracts, they have been utilized for preparing dextrans for commercial use. The production and properties of *Leuconostoc* species dextransucrases have formed the subject of articles by Hehre,²²⁵ Neely,⁶ Tsuchiya,²²⁶ and Ebert and Schenk.²¹⁸

Several species of streptococci are reported^{29, 48, 217, 227–229} to produce dextransucrases, although in many instances the D-glucans synthesized by these enzyme preparations have not been rigorously characterized as dextrans. The properties of streptococcal dextransucrases are somewhat different from those of *Leuconostoc* species: synthetic activities are exhibited over a pH range of 5–8.5, and between 37 and 45°. Above all, the enzymes are constitutive and may, therefore, be obtained free from contamination by dextran.

Most streptococci that elaborate dextransucrases appear to secrete the enzyme(s) exocellularly, although some strains of *Streptococcus mutans* also elaborate structure-bound forms of the enzyme. The structure-bound enzyme activities of *S. mutans* may be situated at several points on the outer surface of the cell wall.²³⁰ Evidence has also been presented that suggests that the proportions of the structure-bound and secreted forms of the enzyme produced by individual streptococci may change with repeated subculturing of the bacteria.²³⁰ Similar changes have not been detected in *Leuconostoc* strains.⁴²

McCabe and Smith^{230a} have observed that the cell-associated dextransucrase elaborated by *S. mutans* strain K1-R is not bound to the cell-wall structure, but is derived from a water-soluble form of the enzyme that becomes incorporated into the (dextran) cell-capsule during dextran synthesis. As synthesis of dextran proceeds, the initially soluble enzyme is converted into two dextran-bound enzyme fractions, one bound reversibly and the other bound irreversibly to the water-insoluble polysaccharide. The soluble dextransucrase is progressively changed to the irreversibly bound enzyme and is inactivated. In terms of the reaction

(225) E. J. Hehre, *Methods Enzymol.*, **1**, 178 (1955).

(226) H. M. Tsuchiya, *Bull. Soc. Chim. Biol.*, **42**, 1777 (1960).

(227) R. W. Bailey, *Biochem. J.*, **72**, 42 (1959).

(228) J. Cybulska and R. Pakula, *Exp. Med. Microbiol.*, **15**, 187 (1963).

(229) E. Newbrun, *Caries Res.*, **5**, 124 (1971).

(230) R. J. Gibbons and R. J. Fitzgerald, *J. Bacteriol.*, **98**, 341 (1969).

(230a) M. M. McCabe and E. E. Smith, *Infect. Immunity*, **7**, 829 (1973).

mechanisms discussed on pp. 427-433, it is tempting to speculate that the reversibly and irreversibly bound enzyme-fractions are constituted of enzyme molecules that are participating, respectively, in the (multi-chain) synthesis of branches or oligosaccharides (see p. 432) and the (single-chain) synthesis of skeletal chains (see p. 428). The gradual formation of the irreversibly bound enzyme may be a consequence of the fact that the dextranucrase-dextran complex (see p. 429) is not dissociated on completion of skeletal-chain synthesis.

Bailey²²⁷ obtained dextranucrases from cultures of a rumen strain of *S. bovis* grown on a series of carbohydrates in the presence of carbon dioxide. A preparation of the enzyme, purified by precipitation from a D-glucose medium with ammonium sulfate, contained less than 4% of dextran, and appeared to synthesize a (1→3)-branched dextran from sucrose.¹⁰⁸

Cybulska and Pakula^{29,228} investigated the effects of varying the composition of the growth medium on the formation of the enzyme by strains of *Streptococcus challis* and *S. sanguis*. Optimal production of the enzymes was achieved by culturing the bacteria on a dialyzed medium containing 1% of D-glucose.²⁹ Crude enzyme-preparations from these sources synthesized D-glucans, containing 50% of (1→6)-linked D-glucopyranosyl residues, that were serologically identified as dextrans.²²⁸ Paper electrophoresis²²⁸ revealed that the synthetic activities of the crude preparations were derived from at least three separate enzymes.

Three enzymes, described as dextranucrases, have also been separated,^{217,229} by chromatography on hydroxylapatite and isoelectric focusing, from the supernatant liquor of a D-glucose-containing culture of *S. sanguis* strain 804. The principal glucosyltransferase activity was associated with an enzyme having²²⁹ an isoelectric pH of 7.9. The enzyme synthesized a water-insoluble dextran containing a high proportion of (1→3)-linked α -D-glucopyranosyl residues.¹²⁶ Sucrose acted as its own primer for dextran synthesis, the extent of which was proportional to the incubation time.²³¹ The rate of D-glucosyl transfer was not, however, proportional to the concentration of the enzyme, and could be increased significantly by adding dextran to the reaction mixture.²³¹ No particular metal-ion requirement was evident.²³¹

Dextranucrases are thought to be elaborated by several strains of *S. mutans*^{48,232-234} that constitute a part of human oral microflora. Gug-

(231) E. Newbrun and J. Carlsson, *Arch. Oral Biol.*, **14**, 461 (1969).

(232) J. M. Wood, *Arch. Oral Biol.*, **12**, 1659 (1967).

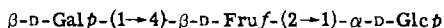
(233) R. J. Gibbons and M. Nygaard, *Arch. Oral Biol.*, **13**, 1249 (1968).

(234) J. M. Tanzer, W. I. Wood, and M. I. Krichevsky, *J. Gen. Microbiol.*, **58**, 125 (1969).

genheim and Newbrun⁴⁸ determined that the exocellular D-glucosyltransferase activity of *S. mutans* OMZ 176 is compounded of the activities of at least six, antigenically distinct enzymes. Members of this family of D-glucosyltransferases synthesize, from sucrose, water-insoluble D-glucans that contain large numbers of (1→3)-linked α-D-glucopyranosyl residues per molecule.¹²⁶ At least one of these D-glucans may be classifiable as a dextran.

(ii) **The Catalytic Activity of Dextransucrase.** It is convenient to regard the catalytic activity of a dextransucrase as a combination of specific reactions involving substrate and acceptor molecules at adjacent, active sites on the enzyme. Three activities are associated with the substrate (or donor) site of a dextransucrase, namely, substrate binding, substrate splitting (sucrase activity), and the transfer of α-D-glucopyranosyl groups (individually or, possibly, in blocks) to an acceptor molecule.

Sucrose is the natural substrate for dextransucrases, although lactulosucrose (66) and α-D-glucopyranosyl fluoride are also reported to act



Lactulosucrose

66

as substrates for dextran synthesis.^{212,219} The sucrose molecule is 1.5 to 3 times as effective as lactulosucrose in this role, from which it may be assumed that the substrate site is tailored closely to the particular stereochemistry of the sucrose molecule.

As a systematic study of the substrate effectiveness of monosubstituted sucrose molecules has not been reported, the minimal stereochemical requirements for substrate activity are uncertain; it would seem, however, that the presence of unsubstituted hydroxyl groups at C-1, C-3, and C-6 of the β-D-fructofuranosyl ring of the sucrose molecule is an essential requirement.^{219,235}

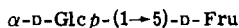
A dextransucrase preparation from *Leuconostoc mesenteroides* NRRL B-1375 has been shown²³⁶ to transfer α-D-glucopyranosyl groups from unlabelled sucrose to the anomeric position of carbon-14 labelled D-fructofuranose (see reaction 2). The rate at which the carbon-14 labelled



(235) K. H. Ebert, G. Rupprecht, and G. Schenk, *Z. Naturforsch. (B)*, **18**, 442 (1963).

(236) E. J. Bourne, J. Peters, and H. Weigel, *J. Chem. Soc.*, 4605 (1964).

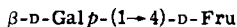
sucrose was synthesized proved to be considerably greater than that at which leucrose-¹⁴C (67) was produced in the same system, and, more-



Leucrose

67

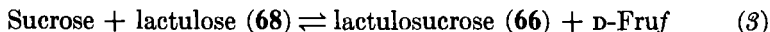
over, was independent of the dextran-synthesizing reaction, as is the synthesis²¹⁹ of lactulosucrose (66) from lactulose (68) and sucrose (see



Lactulose

68

reaction 3). These findings suggested that reactions 2 and 3 may involve

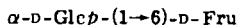


reversible interchanges at the substrate site, rather than transfers of α -D-glucopyranosyl groups to the acceptor site, and that, consequently, both the binding and splitting of sucrose and lactulosucrose at the substrate site of a dextranucrase are reversible processes.

The transfer of α -D-glucopyranosyl groups to a suitable molecule bound at the acceptor site is, apparently, irreversible, as dextranucrase preparations are not found to synthesize sucrose from dextrans plus D-fructose.^{220,237}

Neely⁸ proposed that three activities characterize the acceptor (or receptor) site of dextranucrase, namely, binding of an acceptor molecule, transfer of an α -D-glucopyranosyl group to the acceptor molecule, and the extrusion of the D-glucopyranosyl acceptor to an adjacent site on the enzyme (dextran synthesis) or into solution (oligosaccharide synthesis).

Synthesis of oligosaccharides from sucrose and simple sugars (see Table VIII) demonstrated that the acceptor specificity of dextranu-



Isomaltulose

69

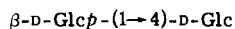
crases is exceptionally broad; it has been proposed that a pyranoid sugar in which there are a minimum of two hydrogen atoms and one

(237) E. J. Hehre, *J. Biol. Chem.*, **162**, 221 (1946).

TABLE VIII
Oligosaccharides Synthesized from Sucrose and Acceptor Molecules by Dextranucrases

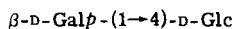
<i>Acceptor</i>	<i>α-D-Glucopyranosyl acceptor</i>	<i>References</i>
D-Fructose	isomaltulose (69)	238-241
D-Fructose	leucrose (67)	238,242
D-Galactose	α -D-Glcp- β -D-Galf	243,244
D-Glucose	isomalto-oligosaccharides (46)	216,222,245,246
D-Mannose	α -D-Glcp- β -D-Manp	244
Cellobiose (70)	β -D-Glcp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)]-D-Glc	247-249
Isomaltose (1)	isomalto-oligosaccharides (46)	216,246,250
Isomaltulose (69)	α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)-D-Fru	238
Lactose (71)	β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)]-D-Glc	248,249,251
Leucrose (67)	α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 5)-D-Fru	238,250
Maltose (4)	panose (39)	222,246,250,252
Raffinose (72)	β -D-Galp-(1 \rightarrow 6)-[α -D-Glcp-(1 \rightarrow 2)]- α -D-Glcp-(1 \rightarrow 2)- β -D-Fruf	253
Theanderose (73)	α -D-Glcp-(1 \rightarrow 6)-[α -D-Glcp-(1 \rightarrow 6)]- α -D-Glcp-(1 \rightarrow 2)- β -D-Fruf	246,254

oxygen atom *cis*-related at C-1, C-3, and C-5 may function as an acceptor molecule.²³⁶ However, the most effective acceptors of transferred α -D-glucopyranosyl groups appear to be molecules that contain one or more α -D-glucopyranosyl residues.²⁵⁰ Results of experiments^{247-249,251} with cellobiose (70) and lactose (71) indicated that dextranucrases do not



Cellobiose

70



Lactose

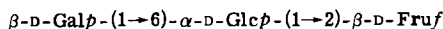
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transfer α -D-glucopyranosyl groups to β -D-glucopyranosyl residues. The equilibrium existing between the α and β anomers of the sugar might account for the observation that D-glucose is a less effective acceptor molecule than methyl α -D-glucopyranoside.^{216,218,222}

Although the effects of introducing substituents onto the α -D-glucopyranosyl residues of acceptor molecules have not yet been systematically investigated, the results of a limited number of experiments employing dextranucrase preparations that synthesize (1 \rightarrow 3)-branched dextrans deserve comment. The substitution of a variety of groups, for example,

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- (238) E. J. Bourne, D. H. Hutson, and H. Weigel, *Biochem. J.*, **79**, 549 (1961).
 - (239) R. Weidenhagen and S. Lorenz, *Angew. Chem.*, **69**, 641 (1957).
 - (240) E. S. Sharpe, F. H. Stodola, and H. J. Koepsell, *J. Org. Chem.*, **25**, 1062 (1960).
 - (241) W. Mauch and S. Schmidt-Lorenz, *Z. Zuckerind.*, **14**, 309 (1964).
 - (242) F. H. Stodola, E. S. Sharpe, and H. J. Koepsell, *J. Amer. Chem. Soc.*, **78**, 2514 (1956).
 - (243) E. J. Bourne, J. Hartigan, and H. Weigel, *J. Chem. Soc.*, 1088 (1961).
 - (244) Y. Iriki and E. J. Hehre, *Arch. Biochem. Biophys.*, **134**, 130 (1969).
 - (245) R. W. Bailey, S. A. Barker, E. J. Bourne, and M. Stacey, *Nature*, **175**, 635 (1955).
 - (246) G. J. Walker, *J. Dent. Res.*, **51**, 409 (1972).
 - (247) S. A. Barker, E. J. Bourne, P. M. Grant, and M. Stacey, *Nature*, **178**, 1221 (1956).
 - (248) R. W. Bailey, S. A. Barker, E. J. Bourne, and M. Stacey, *Nature*, **176**, 1164 (1955).
 - (249) R. W. Bailey, S. A. Barker, E. J. Bourne, P. M. Grant, and M. Stacey, *J. Chem. Soc.*, 1895 (1958).
 - (250) H. J. Koepsell, H. M. Tsuchiya, N. N. Hellman, A. Kazenko, C. A. Hoffman, E. S. Sharpe, and R. W. Jackson, *J. Biol. Chem.*, **200**, 793 (1953).
 - (251) E. J. Bourne, J. Hartigan, and H. Weigel, *J. Chem. Soc.*, 2332 (1959).
 - (252) M. Killey, R. J. Dimler, and J. E. Cluskey, *J. Amer. Chem. Soc.*, **77**, 3315 (1955).
 - (253) W. B. Neely, *Arch. Biochem. Biophys.*, **79**, 154 (1959).
 - (254) R. W. Bailey, S. A. Barker, E. J. Bourne, M. Stacey, and O. Theander, *Nature*, **179**, 310 (1957).

methyl, D-fructofuranosyl, or D-glucopyranosyl, at the anomeric position of the α -D-glucopyranosyl residue has a marked effect on the rate of transfer of an α -D-glucopyranosyl group to this acceptor residue, but, in each case, the group is transferred^{218,250,255} to the primary hydroxyl group on C-6. If only the secondary, 4-hydroxyl group is substituted [as in cellobiose (70) or lactose (71)], the α -D-glucopyranosyl group is transferred to O-2 of the D-glucose residue (bound presumably in its α -anomeric configuration) to form a branched trisaccharide.^{247-249,251} The transferred α -D-glucopyranosyl group also enters²⁵³ at O-2 of the O-1, O-6-substituted α -D-glucopyranosyl residue of raffinose (72), and at O-6



Raffinose

72

of 3-O-methyl-D-glucose.²⁵⁶ On the basis of these findings, it might be speculated that (a) the position of the substituent in the acceptor α -D-glucopyranosyl residue determines the orientation of the acceptor molecule in the acceptor site, and, hence, the hydroxyl group to which the α -D-glucopyranosyl group is transferred, and (b) the nature of the substituent, although affecting the rate at which the α -D-glucopyranosyl group is transferred to the acceptor residue, may have little effect in determining the hydroxyl group to which the α -D-glucopyranosyl group is transferred. If dextranases are the only enzymes catalyzing branched-dextran synthesis, substituent effects of this kind, together with structural differences in the acceptor sites of individual dextranases, may account for both the ratio of the secondary to the (1 \rightarrow 6)- α -D-glucopyranosidic linkages, and the types of secondary linkages found in different dextrans.

2. Mechanism of Synthesis

The structural studies discussed in Section III demonstrate that two reactions are of fundamental importance in dextran synthesis: the formation of skeletal chains composed of (1 \rightarrow 6)-linked α -D-glucopyranosyl residues, and the introduction of branches into these chains.

Synthesis of the skeletal chains must proceed, as do all enzyme-controlled polymerization reactions, either by a single or a multichain reaction mechanism, through a series of "growing" cycles in which the

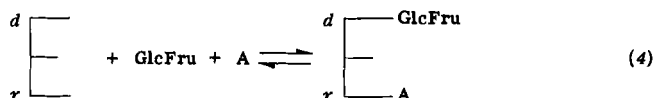
(255) K. H. Ebert and G. Schenk, *Z. Naturforsch. (B)*, **23**, 788 (1968).

(256) R. W. Bailey, S. A. Barker, E. J. Bourne, P. M. Grant, and M. Stacey, *J. Chem. Soc.*, 601 (1958).

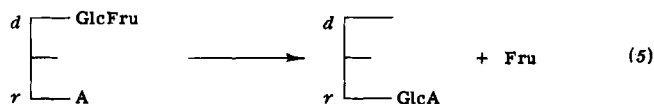
enzyme catalyzes the addition of a D-glucopyranosyl (or larger) group to the growing dextran chain. If a multichain mechanism operates, the complex that forms between the enzyme and the dextran chain will dissociate at the end of each growing cycle, enabling several enzyme molecules to participate in the formation of a single skeletal chain. In the event of a single-chain mechanism, each skeletal chain will be synthesized by a single enzyme molecule, dissociation (or inactivation) of the enzyme-dextran complex terminating chain synthesis after several growing cycles.

Failure to detect oligosaccharides in the early stages of dextran synthesis²⁴⁶ and the observation that dextrans attain high molecular weights ($\bar{M}_w \sim 5 \times 10^6$) in the early stages of the synthetic reaction^{196,267} have been cited as evidence that a single-chain mechanism operates during the synthesis of skeletal chains.

As, in theory, the structural polarity of dextran molecules allows skeletal chains to be formed by transfers of D-glucopyranosyl groups to either the reducing or nonreducing end of these chains, two distinct, single-chain mechanisms of skeletal chain-growth are possible. By assuming that skeletal chains grow from their nonreducing ends, Neely⁸ proposed that chain formation might involve the following reactions: (a) the simultaneous binding of a sucrose molecule (GlcFru) and a sucrose acceptor molecule (A) at donor (d) and receptor (r) sites, respectively, of dextranucrase (E) (see equation 4); (b) the transfer of a D-glucopyranosyl group (Glc) from the substrate to the acceptor molecule, with

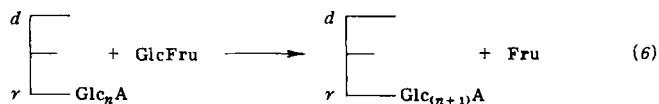


release of a D-fructose molecule (Fru) (see reaction 5); (c) the rapid

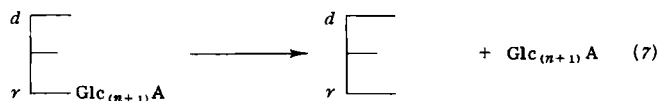


transfer of D-glucopyranosyl groups from a succession of substrate molecules to the primary hydroxyl group of the terminal, D-glucopyranosyl group of the growing skeletal chain (Glc_nA) (see reaction 6), and (d)

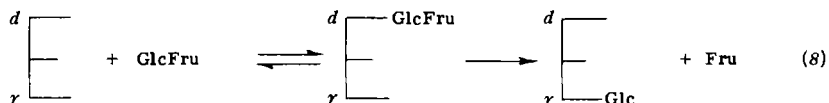
(257) H. M. Tsuchiya, N. N. Hellman, H. J. Koepsell, J. Corman, C. S. Stringer, S. P. Rogovin, M. O. Bogard, G. Bryant, V. H. Feger, C. A. Hoffman, F. R. Senti, and R. W. Jackson, *J. Amer. Chem. Soc.*, **77**, 2412 (1955).



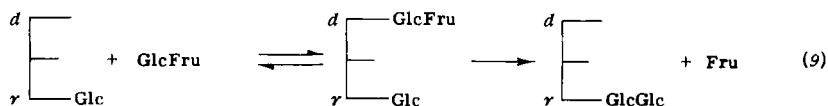
the nonspecific dissociation (or inactivation) of the dextran-dextran-sucrase complex, which terminates chain synthesis (see reaction 7). An



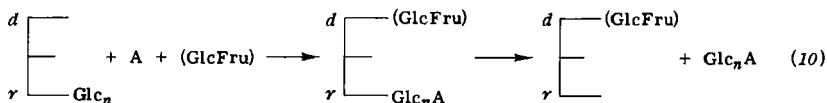
alternative mechanism was proposed by Ebert and Schenk;²¹⁸ it assumed that skeletal chains grow from their reducing ends. In this scheme, it was suggested that the following sequence of reactions leads to chain formation: (a) a sucrose molecule (GlcFru) enters the dextran-sucrase donor-site (*d*) and is hydrolyzed, and the D-glucopyranosyl group (Glc) so released is transferred to the receptor site (*r*) of the enzyme (see equation 8); (b) a second sucrose molecule enters the now-vacant donor



site and is hydrolyzed, and chain synthesis is initiated by transfer of the released D-glucopyranosyl group to the anomeric (C-1) position of the D-glucopyranosyl group in the receptor site (see equation 9); a series of

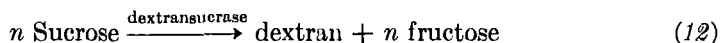
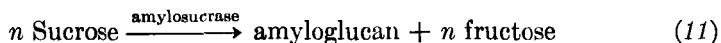


identical steps propagates the chain until (c) the synthesis is terminated by the nonspecific reaction of an acceptor molecule (A) with the skeletal chain (Glc_n) (see reaction 10).



Present experimental evidence (reviewed in articles by Hehre,^{221,258}

Neely,⁶ Tsuchiya,²²⁶ and Ebert and Schenk²¹⁸) provides no direct indication of the direction of skeletal chain-propagation, and, hence, cannot be used to test the validity of these mechanisms. Hehre²⁵⁹ pointed out, however, that the direction of chain propagation envisaged by Ebert and Schenk²¹⁸ is contrary to that considered usual for D-glucosyltransferases. Hehre,²⁵⁹ moreover, demonstrated (by a pulse-labelling technique) that the latest D-glucopyranosyl groups transferred from sucrose to *Neisseria perflava* amyloglucan by amylosucrase are located within terminal, non-reducing sequences of the skeletal chains; this observation suggested that the amyloglucan is synthesized by transfer of D-glucopyranosyl groups to nonreducing ends of the skeletal chains, and that, as the catalytic activities of amylosucrases and dextransucrases are comparable (see reactions 11 and 12), dextran chains are formed in this way.



The uncertainty that exists in our understanding of the precise nature of the role played by dextransucrases in branched-dextran synthesis (see p. 418) is well reflected in the numerous mechanisms of branch-formation that have thus far been formulated.

In an early proposal, Bovey suggested that branches consisting of single D-glucopyranosyl groups might be formed by the transfer of D-glucopyranosyl groups from sucrose molecules,²⁶⁰ and that an enzyme other than dextransucrase might synthesize branches of greater length by the scission and rearrangement of linear skeletal chains.¹⁹⁶ The latter proposal stemmed from light-scattering and periodate-oxidation measurements that suggested that branched dextrans having high molecular weights ($\bar{M}_w \sim 5 \times 10^7$) are formed during the early stages of the synthetic reaction and, in particular, that the molecular weights of dextrans continue to increase after all of the sucrose substrate has been utilized.¹⁹⁶

In commenting on this proposal, Hehre²⁵⁸ observed that, in Nature, the incidence of transglycosylases catalyzing the transfer of chain segments from primary to secondary hydroxyl groups is rare, if, indeed, the process occurs at all, and that it must be considered doubtful whether sufficient free energy would be released by this reaction to account for the high percentage of secondary linkages present in some dextrans. Significantly, enzymes that specifically catalyze skeletal chain rearrangements have not been isolated from dextran fermentation-mixtures.

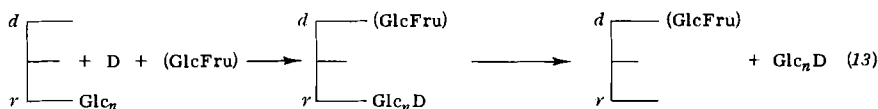
On applying several independent methods to determination of the

(259) E. J. Hehre, *J. Polym. Sci., Part C*, **23**, 239 (1968).

(260) F. A. Bovey, *J. Polym. Sci.*, **35**, 191 (1959).

molecular weight of native dextrans, Ebert and coworkers⁸⁰ concluded that the highest molecular weights reported²⁶⁰ by Bovey ($\bar{M}_w \sim 2$ to 5×10^8) are particle weights of associations of dextrans having molecular weights (\bar{M}_w) in the order of 3 to 5×10^5 . This finding suggested that the increases in "molecular weight" that are reported to occur in the absence of sucrose (and that have been attributed to branch-forming rearrangements of linear chains) may be largely increases in particle weight brought about by the increasing association of dextrans of constant molecular weight.

The observation that dextran molecules are good acceptors of transferred D-glucopyranosyl groups^{231, 261-263} prompted Ebert and Brosche²⁶³ to suggest that branches are formed by a reaction analogous to the non-specific, acceptor reaction that may terminate skeletal-chain synthesis (see equation 10). When the acceptor terminating growth of a skeletal chain is a dextran molecule, the skeletal chain (Glc_n) will most frequently be transferred to a secondary hydroxyl group in the acceptor dextran (D), to form a branch according to reaction 13. In the resulting



branched dextran ($\text{Glc}_n D$), Glc_n constitutes the branch-chain in respect of the branch formed in this reaction.

A possible criticism of this mechanism of branch formation derives from the fact that its operation should result in the synthesis of a dextran in which the lengths of the branches are randomly distributed, whereas most of the branches in many native dextrans (see Section III, p. 406) appear to consist either of short sequences of D-glucopyranosyl residues, or single D-glucopyranosyl groups. In addition, indirect evidence has been presented by Hehre²⁵⁹ that indicates that the mode of formation of the branch chains envisaged in this proposal (by transfers of D-glucopyranosyl groups to the reducing ends of the chains; see p. 429) may be incorrect.

A third mechanism of branch formation has been proposed by Hehre.²⁵⁹ In this scheme, the author assumed that branches are built up exclusively by transfers of D-glucopyranosyl groups from sucrose to the nonreducing ends of growing branch-chains, and he ascribed the rapid synthesis of

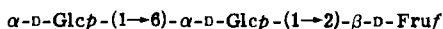
(261) H. M. Tsuchiya, N. N. Hellman, and H. J. Koepsell, *J. Amer. Chem. Soc.*, **75**, 757 (1953).

(262) E. J. Hehre, *J. Amer. Chem. Soc.*, **75**, 4866 (1953).

(263) K. H. Ebert and M. Brosche, *Biopolymers*, **5**, 423 (1967).

dextrans of high molecular weight to the simultaneous propagation of several branch-chains.

Although a detailed mechanism was not formulated, the underlying concept, namely, branch formation by rapid, sequential transfers of D-glucopyranosyl groups, is open to question. In this proposal, branch synthesis must be initiated by the transfer of a D-glucopyranosyl group to an acceptor D-glucopyranosyl residue within a skeletal chain. The results of experiments in which a wide range of acceptor molecules were added to dextran-synthesizing systems indicated that the initial (and any subsequent) transfers of D-glucopyranosyl groups to acceptors other than sucrose proceed by way of a multi-chain reaction-mechanism^{254,260,264} (see p. 427), and result in the formation of short-chain oligosaccharides (see Table VIII, p. 425), not of dextrans of high molecular weight. Thus, if branches are formed by sequential transfers of D-glucopyranosyl residues to dextran chains, this experience suggested that these branches will not attain great length, and that their synthesis involves a slow, multi-chain reaction. Furthermore, the scheme of branch formation envisaged by Hehre²⁵⁹ is not compatible with the fact that the great majority of branches in native dextrans appear to consist of no more than one or two D-glucopyranosyl residues, and that only a very small number of branches attain great lengths (see pp. 406-411). This pattern of branch-length distribution, indeed, suggests that two mechanisms of branch formation operate during dextran synthesis. As mentioned earlier (see p. 430), short branches may be built up by multi-chain transfers of D-glucopyranosyl groups from sucrose substrates to skeletal chains; the formation of long branches, in contrast, probably involves some form of segment transfer. Conceivably, the segments transferred are skeletal chains that are "capped" at the reducing end by a β -D-fructofuranosyl group,^{258,265} although two theoretically appropriate substrates for such a reaction, namely, the trisaccharide theanderose (73) and β -D-fructo-



Theanderose

73

furanosyl-(2 \rightarrow 1)-dextran, do not exhibit donor activity when incubated with dextranucrase preparations.^{254,259} It is possible, however, that these preformed molecules differ conformationally from equivalent molecules formed *in situ* from sucrose, particularly as the latter are most likely to

(264) B. Weibull, *Acta Chem. Scand.*, **12**, 568 (1958).

(265) C. S. Stringer and H. M. Tsuchiya, *J. Amer. Chem. Soc.*, **80**, 6620 (1958).

be bound to the synthesizing enzyme, and, as a result, may be unable to enter the dextransucrase donor-site.

V. MISCELLANEOUS

1. Dextrans and Dental Caries

Considerable evidence now exists to indict dietary sucrose as the agent that initiates dental caries (an infective disease that breaks down tooth enamel and dentine) in persons living in technologically advanced communities.²⁶⁶ The extreme cariogenicity of sucrose appears to be related to its ability to act as a substrate for the synthesis of a number of exocellular, bacterial polysaccharides, including dextrans and levans. The significant role that oral dextrans are believed to play in the development of dental caries, and the possibility of controlling tooth decay by preventing the synthesis of these dextrans, are examined in the present Section.

a. Formation of Dental Plaque.—It is now generally recognized that caries lesions are caused by organic acids that are produced by oral bacteria from fermentable carbohydrates.²⁶⁷⁻²⁷¹ In molar teeth, caries may develop in occlusal fissures as a result of bacterial fermentation of impacted food-materials. However, lesions on the more numerous "self-cleansing," smooth surfaces of teeth cannot form in a similar way. A prerequisite to the development of smooth-surface lesions appears to be the accumulation of a layer of polysaccharide-containing plaque on the tooth surface.^{272,273}

It would seem that the role of plaque in the etiology of dental caries is threefold: to provide (*a*) a stable matrix and (*b*) a source of fermentable carbohydrate for acidogenic bacteria,²⁶⁷⁻²⁷¹ and (*c*) a diffusion-

(266) (a) E. Newbrun, *Odontol. Revy*, **18**, 370 (1967). (b) T. H. Grenby, *Chem. Brit.*, **7**, 276 (1971). (c) K. K. Mäkinen, *Int. Dent. J.*, **22**, 363 (1972).

(267) R. J. Fitzgerald, H. V. Jordan, and H. O. Archard, *Arch. Oral Biol.*, **11**, 473 (1966).

(268) K. S. Berman and R. J. Gibbons, *Arch. Oral Biol.*, **11**, 533 (1966).

(269) J. Van Houte, K. C. Winkler, and H. M. Jansen, *Arch. Oral Biol.*, **14**, 45 (1969).

(270) D. B. Drucker and T. H. Melville, *Arch. Oral Biol.*, **16**, 845 (1971).

(271) G. Hu and H. J. Sandham, *Arch. Oral Biol.*, **17**, 729 (1972).

(272) B. Krasse, *Arch. Oral Biol.*, **10**, 223 (1965).

(273) (a) R. J. Fitzgerald and H. V. Jordan, in "Art and Science of Dental Caries Research," R. S. Harris, ed., Academic Press, New York, 1968, p. 79. (b) R. J. Gibbons, *ibid.*, p. 121.

restricting barrier²⁷⁴⁻²⁷⁶ that enables^{277,278} a low pH to be maintained at the tooth surface.

The mode of formation and characteristics of dental plaques formed under different conditions will be considered briefly at this stage in order to facilitate later discussions. Immediately a cleaned tooth is exposed to the oral environment, an organic film begins to form on the tooth surface adjacent to the gingiva (gum margin).²⁷⁹ After a period of several hours, this film, termed acquired pellicle, may cover most of the tooth surface.^{279,280} The composition of the pellicle indicates that it is formed from deposited salivary and serum proteins,²⁸¹⁻²⁸⁴ the latter being present in crevicular fluids that are secreted from the gingival tissues.²⁸¹ The formation of pellicle represents the first stage of plaque development.

Within minutes of the onset of pellicle formation, aggregates of bacteria begin to adhere to the pellicle at several sites on the tooth adjacent to the gingiva, and are later to be seen colonizing other sheltered areas of the tooth surface.²⁷⁹ Under the electron microscope, these bacterial aggregates appear initially as isolated hemispherical globules²⁷⁹ that increase in size, and eventually coalesce to form a continuous layer termed dental plaque.

The same basic sequence of events accompanies pellicle- and plaque-formation irrespective of whether food is present or absent. However, plaques formed under these two conditions usually exhibit significant morphological and physiological differences.²⁸⁰ A thin layer of plaque that, for convenience, will be termed fasting plaque, is formed in the absence of food;²⁸⁰ this kind of plaque is composed largely of bacteria, and has a protein matrix. The pH of fasting plaque is often higher than that of the surrounding saliva²⁸⁵ and, in consequence, the plaque is not

(274) L. M. Silverstone, *Brit. Dent. J.*, **120**, 461 (1966).

(275) P. Critchley, J. M. Wood, C. A. Saxton, and S. A. Leach, *Caries Res.*, **1**, 112 (1967).

(276) C. A. Saxton, *Arch. Oral Biol.*, **14**, 1275 (1969).

(277) H. Graf and H. R. Mühlemann, *Helv. Odont. Acta*, **10**, 94 (1966).

(278) J. De Boever and H. R. Mühlemann, *Helv. Odont. Acta*, **13**, 97 (1969).

(279) C. A. Saxton, *Caries Res.*, **7**, 102 (1973).

(280) J. Carlsson and J. Egelberg, *Odontol. Revy*, **16**, 112 (1965).

(281) E. Weinstein and I. D. Mandel, *J. Amer. Soc. Periodont.*, **2**, 147 (1964).

(282) W. G. Armstrong, *Nature*, **210**, 197 (1966).

(283) S. A. Leach, P. Critchley, A. B. Kolendo, and C. A. Saxton, *Caries Res.*, **1**, 104 (1967).

(284) T. Sönju and G. Rölla, *Caries Res.*, **7**, 30 (1973).

(285) G. N. Jenkins, *Int. Dent. J.*, **22**, 350 (1972).

cariogenic. Plaque formed in the presence of the components of a normal diet is more voluminous than fasting plaque.²⁸⁰ The increase of volume is largely accounted for by the incorporation of polysaccharides into the plaque matrix.^{276,280,286,287} Moreover, the pH of the plaque^{277,278} is usually somewhat lower than that of fasting plaque, and acidic secretions from the plaque may cause the tooth enamel beneath the plaque to become hypomineralized. In cases of excessive hypomineralization, the tooth structure is irreparably damaged and a typical caries lesion is formed.^{274,288-291} The plaque is then described as cariogenic.

Diets containing high levels of sucrose promote the formation of plaques that contain unusually large proportions of matrix polysaccharides^{276,280,286,287,290} beneath which caries lesions rapidly develop.²⁷² A large proportion of the matrix polysaccharides may be extracted from sucrose-derived plaques with water or aqueous alkali,^{20,275,292,293} and they have been found to consist largely of D-glucans and D-fructans. In pure culture, bacteria isolated from cariogenic plaques synthesize abundant amounts of exocellular D-glucans or D-fructans, or both, from sucrose,^{20,35,37,52} but not from other carbohydrates,^{20,35,47,217,294} suggesting that these matrix polysaccharides are synthesized *in vivo* by plaque bacteria from dietary sucrose.^{20,272,275,276,280,292,294,295} Structural analysis of the exocellular D-glucans indicated that many of them are dextrans.^{20,35,36,110,123,123a,126}

b. The Role of Dextrans in Caries Development.—Although there can be little doubt that dextrans are implicated in the development of sucrose-induced dental caries, the precise role that these polysaccharides play in the cariogenic process is, as yet, uncertain. Available evidence, however, suggests that dextrans promote dental caries by facilitating the colonization of cariogenic bacteria on the tooth surface and through their presence as structural components, or reserve carbohydrates, in cariogenic plaques. Each of these propositions will be examined in turn.

- (286) J. Carlsson and B. Sunderström, *Odontol. Revy*, **19**, 161 (1968).
- (287) P. Critchley, C. A. Saxton, and A. B. Kolendo, *Caries Res.*, **2**, 115 (1968).
- (288) F. von Bartheld, *Arch. Oral Biol.*, **6**, 284 (1961).
- (289) M. D. Francis and A. H. Meckel, *Arch. Oral Biol.*, **8**, 1 (1962).
- (290) P. Critchley, *Caries Res.*, **3**, 249 (1969).
- (291) J. M. Hardie, L. M. Silverstone, and G. H. Bowden, *Caries Res.*, **5**, 290 (1971).
- (292) J. Carlsson, *Odontol. Revy*, **16**, 348 (1965).
- (293) S. A. Leach, R. M. Green, M. L. Hayes, and O. A. Dada, *J. Dent. Res.*, **48**, 811 (1969).
- (294) J. H. Shaw, I. Krumins, and R. J. Gibbons, *Arch. Oral Biol.*, **12**, 755 (1967).
- (295) R. J. Gibbons, K. S. Berman, P. Knoettner, and B. Kapsimalis, *Arch. Oral Biol.*, **11**, 549 (1966).

(i) **The Bacterial Colonization of the Tooth Surface.** Bacteria of the genus *Streptococcus* form an important part of the human microflora,^{296,297} and are the largest group of micro-organisms colonizing early dental plaque.^{280,298} The predominant group of streptococci in dental plaques are strains of *Streptococcus sanguis*,²⁹² although a considerable part of the streptococcal population of cariogenic (sucrose-derived) plaques comprises *S. mutans*.^{299,300} The presence of large numbers of *S. mutans* in cariogenic plaques, and their association with caries lesions in both man and animals,^{295,300-305} resulted in a belief that *S. mutans* are key micro-organisms in the cariogenic process in man. Prompted by this belief, many properties of *S. mutans* have been investigated, including the manner in which the bacteria colonize the tooth surface.

As mentioned earlier (see p. 434), bacterial colonization of a tooth surface is dependent upon the bacteria's forming adherent aggregates on the tooth pellicle or dental plaque. Whereas many bacteria form these adherent aggregates in the presence of salivary components,^{306,307} *S. mutans* requires the presence of dietary sucrose.³⁰⁷⁻³¹⁰ Gibbons and Fitzgerald investigated the mechanism by which *S. mutans* forms aggregates in the presence of sucrose.²³⁰ Two reactions appear to be involved: firstly, the dextransucrase-mediated synthesis of dextrans from sucrose and, secondly, the binding, through their cell-bound dextransucrases, of several *S. mutans* cells to common (cross-linked³¹⁰) dextrans of high molecular weight.

On the basis of these observations, it may be visualized that a dextran-

- (296) J. Carlsson, *Odontol. Revy*, **19**, 1 (1968).
- (297) J. Carlsson, G. Söderholm, and I. Almfeldt, *Arch. Oral Biol.*, **14**, 243 (1969).
- (298) H. L. Ritz, *Arch. Oral Biol.*, **12**, 1561 (1967).
- (299) J. Carlsson, *Odontol. Revy*, **19**, 137 (1968).
- (300) J. D. de Stoppelaar, J. Van Houte, and O. Backer-Dirks, *Caries Res.*, **4**, 114 (1970).
- (301) D. D. Zinner, J. M. Jablon, A. P. Aran, and H. S. Saslow, *Proc. Soc. Exp. Biol. Med.*, **118**, 766 (1965).
- (302) B. Krasse, *Arch. Oral Biol.*, **11**, 429 (1966).
- (303) N. W. Littleton, S. Kakehashi, and R. J. Fitzgerald, *Arch. Oral Biol.*, **15**, 461 (1970).
- (304) I. L. Shklair, H. J. Keene, and L. G. Simonson, *J. Dent. Res.*, **51**, 882 (1972).
- (305) T. Ikeda, H. J. Sandham, and E. L. Bradley, *Arch. Oral Biol.*, **18**, 555 (1973).
- (306) D. Hay, R. J. Gibbons, and D. M. Spinell, *Caries Res.*, **5**, 111 (1971).
- (307) R. J. Gibbons and D. M. Spinell, in "Dental Plaque," W. D. McHugh, ed., E. and S. Livingston, Edinburgh and London, 1970, p. 207.
- (308) J. D. de Stoppelaar, K. G. Koenig, A. J. M. Plasschaert, and J. S. van der Hoeven, *Arch. Oral Biol.*, **16**, 971 (1971).
- (309) G. A. Olson, A. S. Bleiweiss, and P. A. Small, *Infec. Immunity*, **5**, 419 (1972).
- (310) J. Kelstrup and T. D. Funder-Nielsen, *Arch. Oral Biol.*, **17**, 1659 (1972).

containing plaque on the tooth surface will have the property of being able to select *S. mutans* bacteria from the oral environment and incorporate them into the plaque structure. Moreover, in the presence of dietary sucrose, it appears probable that *S. mutans* cells will elaborate dextran capsules. Specific interactions between these capsular dextrans and the protein components of the pellicle,^{20,311} or tooth material,^{20,309,311} might enable aggregates of the dextran-encapsulated cells to adhere to the tooth surface.

(ii) **Dextrans as Reserve Carbohydrates.** The gradual breakdown of macromolecular, reserve carbohydrates to fermentable sugars in cariogenic plaques is a key factor in the cariogenic process, as it enables a low pH to be maintained at the tooth surface for a prolonged period of time and in the absence of exogenous carbohydrates. Endocellular, glycogen-like polysaccharides form the usual reserve-carbohydrates available to oral bacteria,^{268,269,273,275} although, in dental plaque, exocellular bacterial polysaccharides are important additional reserve-carbohydrates,^{57,273,290,312} because of the diffusion-restricting qualities of plaque (see p. 439). It has been suggested³¹³ that exocellular bacterial dextrans (which constitute a large part of the matrices of cariogenic plaques^{35,275,286}) may form a part of this additional, carbohydrate reserve. Some evidence has been presented to support this view. Wood and associates^{275,280,314,315} established that most of the matrix carbohydrates in mixed, human plaques are dextrans, and demonstrated that up to half the matrix carbohydrates present in similar (24-hour) samples of plaque are metabolized on incubating the samples *in vitro* for 24 hours. Metabolism of so large a proportion of the total, exocellular carbohydrate would necessarily imply that the matrix dextrans are degraded by the plaque bacteria. Moreover, D-glucose and isomaltose (1, p. 378) have been identified in partial acid hydrolyzates of water-soluble carbohydrates of low molecular weight that had been extracted from pooled human plaques;³¹³ this finding suggested that the carbohydrates of low molecular weight may be isomalto-oligosaccharides (46, p. 401) arising from the enzymic breakdown of plaque dextrans. Enzymes (endo- and exo-cellular) that are capable of degrading dextrans of low molecular weight *in vitro* have also been isolated from human saliva and plaque.³¹⁶

- (311) G. Rölla and P. Mathiesen, in "Dental Plaque," W. D. McHugh, ed., E. and S. Livingston, Edinburgh and London, 1970, p. 129.
- (312) J. M. Wood, *J. Dent. Res.*, **43**, 955 (1964).
- (313) P. Hotz, B. Guggenheim, and R. Schmid, *Caries Res.*, **6**, 103 (1972).
- (314) J. M. Wood, *Arch. Oral Biol.*, **12**, 849 (1967).
- (315) J. M. Wood, *Arch. Oral Biol.*, **14**, 161 (1969).
- (316) K. K. Mäkinen and A. Scheinen, *Acta Odont. Scand.*, **30**, 259 (1972).

The inability of mixed plaque-bacteria,^{20,317} or plaque streptococci in pure culture,³¹⁸ to utilize dextrans of high molecular weight as growth substances *in vitro* points to the possibility that the metabolism of plaque dextrans *in vivo* is unlikely to be initiated by enzymes that utilize dextrans as specific substrates, but, instead, may be initiated by enzymes whose primary action is to degrade other plaque-carbohydrates. There are at least two systems of enzymes in dental plaque that could conceivably degrade dextrans. In early plaque, strains of *Neisseria* proliferate,²⁹⁸ and these are capable of synthesizing exocellular, glycogen-like polysaccharides from sucrose.³¹⁹ These polysaccharides are readily degraded *in vitro* by many plaque bacteria.³¹⁸ It is, therefore, possible that the enzymes that hydrolyze the α -D-(1→4)- or α -D-(1→6)-glucopyranosidic linkages, or both, of *Neisseria* polysaccharides also slowly hydrolyze the α -D-(1→6)-linkages of dextrans. Enzymes having this range of substrate activity have been isolated from species of *Aspergillus*,¹⁷⁰ and are present in many animal tissues (see p. 395). In addition, enzymes that normally degrade endocellular, glycogen-like polysaccharides may be released into the plaque matrix following cell lysis; some of these enzymes may be anticipated to exhibit exo- α -D-glucan 6-glucanohydrolase activity.⁶¹

If, as present evidence suggests (see p. 437), dextrans are metabolized in dental plaque, it remains to explain why, *in vitro*, isolated and mixed plaque bacteria are unable to utilize dextrans of high molecular weight as growth substances. A solution to this question might lie in the fact that, *in vivo*, plaque bacteria synthesize both branched dextrans of high molecular weight that are resistant to metabolism, and unbranched dextrans (oligodextrans) of low molecular weight that are readily degraded by plaque enzymes.^{61,310} Structural analysis of carbohydrate extracts from dental plaque supports such a proposal.^{275,290,313,315} Furthermore, the concentrations of carbohydrates of low molecular weight that might be expected to act as alternative acceptors for dextransucrases (see p. 424) would appear to be sufficiently high in dental plaques^{275,290,313-315} to cause substantial amounts of oligodextrans to be synthesized from dietary sucrose.

(iii) **Dextrans as Structural Components of Plaque.** Many plaque bacteria, *in vitro*, synthesize (from sucrose) dextrans that form compacted gels (see p. 417)^{20,37,52,120} in the presence of aqueous solvents at pH values equivalent to those in cariogenic plaques.^{277,278} This finding suggested that dextrans of high molecular weight exist typically as gels in

(317) R. S. Manly and J. A. Dain, *Abstr. Papers Int. Ass. Dent. Res.*, **41**, 125 (1963).

(318) R. D. Parker and H. R. Creamer, *Arch. Oral Biol.*, **10**, 855 (1971).

(319) S. A. Barker, E. J. Bourne, and M. Stacey, *J. Chem. Soc.*, 2884 (1950).

cariogenic dental plaques, and, indeed, electron-microscope examinations revealed that the matrices of plaques formed in the presence of sucrose contain extensive, microfibrillar networks of polysaccharides.^{85,320} As dextrans appear to constitute the major, matrix polysaccharides in sucrose-derived plaques,^{35,275,286,314} there would seem to be little doubt that these microfibrillar structures are dextran-gel networks.

Gels have several distinctive properties;²⁰² they enable a small number of macromolecules to control a large solvent volume, they have stress-resisting structures, and they act as molecular sieves. It is, thus, not difficult to conceive that the presence of an extensive, dextran-gel network would greatly enhance the volume,²⁸⁶ cohesiveness,²⁸⁰ and impermeability (with respect to macromolecules and bacterial cells)^{290,299} of a sucrose-derived plaque.

Dextrans are reported to interact with salivary proteins,^{20,311} certain oral bacteria,^{230,310} and phosphate ions.³¹⁰ It may be envisaged that each of these components could be actively incorporated into the matrix of a plaque that contains a dextran-gel network. The incorporation of proteins and phosphate ions, moreover, would impart a charge to this network; thus, in addition to preventing the free exchange of macromolecules between saliva and the tooth surface, the dextran gel would have the capacity to control the rate at which calcium and phosphate ions leave the tooth surface, and this appears to be an important factor in the formation of natural, subsurface, caries lesions.^{274,288-291} In contrast, the diffusion of small, neutral molecules into plaque does not appear to be prevented by the dextran gel, as electron micrographs of plaques differentially stained for carbohydrates indicated that both endocellular and exocellular reserve-carbohydrates depleted by bacterial metabolism are rapidly re-formed in the presence of dietary sucrose.^{275,287,290}

Because proteins of salivary origin are major constituents both of plaque and tooth pellicle,^{281-284,290} it appears likely that dextran-protein complexes^{20,311} also serve to stabilize the structures of sucrose-derived plaques and bind them to the tooth pellicle.

Studies *in vitro* suggest that, in addition to fulfilling a structural role in dental plaque, dextrans of high molecular weight, acting as inert polymers, also serve to increase the activities of such plaque enzymes as levansucrase and levanhydrolase.^{320a} Although the net effect of this action upon plaque cariogenicity cannot be assessed on present evidence, it may be particularly significant, as levans appear to be important reserve-carbohydrates in cariogenic plaques.^{52,55-59,273}

(320) C. A. Saxton and J. S. Findley, *J. Dent. Res.*, **50**, 1174 (1971).

(320a) J. Kelstrup and T. D. Funder-Nielsen, *Acta Odont. Scand.*, **30**, 621 (1972).

c. **The Prevention of Dental Caries.**—The important role attached to dextrans in the development of dental caries prompted research to ascertain whether the disease can be controlled through the use of agents that might be used clinically to degrade, or prevent the synthesis of, plaque dextrans. The employment of two such agents will be examined.

(i) **Dextranases.** Dextranases are enzymes that cleave the α -D-(1 \rightarrow 6) linkages in dextrans (see p. 391) and might, therefore, if added to food, act as anticaries agents by breaking down the matrices of cariogenic plaques, or by preventing cariogenic bacteria from colonizing the tooth surface (see p. 436). In preliminary, *in vitro* experiments, endodextranases from *Penicillium* species were found to be effective in dispersing both artificial and natural plaques,^{321-322a} and in preventing the sucrose-induced aggregation of cariogenic bacteria (see p. 436).³⁵ To examine the anticaries action of dextranases *in vivo*, the enzymes were incorporated into diets, high in sucrose, fed to caries-susceptible animals, and the incidence of caries in these animals was compared to that in animals fed dextranase-free, but otherwise identical, diets. The enzymes were reported to be effective in inhibiting caries in gnotobiotic animals infected with cariogenic streptococci,^{323,324} but less effective in animals harboring their indigenous microflora.^{325,326} Also, a number of short-term trials have been conducted with human subjects.³²⁷⁻³³⁰ In these experiments, the anticaries effect of the enzymes was assessed by attempting to measure the amounts of plaque remaining on the tooth surfaces after the use of mouthwashes containing the enzyme. Some lessening of plaque was reported within two groups of subjects,^{328,329} but none was observed in other groups.^{327,330}

(321) W. H. Bowen, *Brit. Dent. J.*, **124**, 347 (1968).

(322) R. J. Fitzgerald, D. M. Spinell, and T. H. Stoudt, *Arch. Oral Biol.*, **13**, 125 (1968).

(322a) S. Hoffman, H. D. Tow, and J. S. Cole, *J. Dent. Res.*, **52**, 551 (1973).

(323) R. J. Fitzgerald, P. H. Keyes, T. D. Stoudt, and D. M. Spinell, *J. Amer. Dent. Ass.*, **76**, 301 (1968).

(324) K. G. Koenig and B. Guggenheim, *Helv. Odont. Acta*, **12**, 48 (1968).

(325) B. Guggenheim, K. G. Koenig, H. R. Mühlemann, and B. Regolati, *Arch. Oral Biol.*, **14**, 555 (1969).

(326) W. H. Bowen, *Brit. Dent. J.*, **131**, 445 (1971).

(327) R. C. Cardwell, H. J. Sandham, W. V. Mann, S. B. Finn, and A. J. Formicola, *J. Amer. Dent. Ass.*, **82**, 124 (1971).

(328) R. R. Lobene, *J. Amer. Dent. Ass.*, **82**, 132 (1971).

(329) P. H. Keyes, M. A. Hicks, B. M. Goldman, R. M. McCabe, and R. J. Fitzgerald, *J. Amer. Dent. Ass.*, **82**, 136 (1971).

(330) S. Nyman, J. Lindhe, and J. C. Janson, *Odontol. Revy*, **23**, 243 (1972).

The disappointing results achieved in many of these *in vivo* experiments may be due to the choice of *Penicillium* dextranases, because, *in vitro*, these do not significantly degrade dextrans containing high proportions of secondary-linked, α -D-glucopyranosyl residues,^{49,140} but the results most probably reflect basic structural differences between artificial plaques and plaques formed *in vivo*. In any event, the use of *Penicillium* dextranases as dietary anticaries additives would almost certainly be impractical, owing to their sensitivity to temperature, their low pH optima,¹⁴⁰ and the fact that they have properties that make them inimical to white blood-cells.³²⁶

(ii) **Anticaries Vaccines.** Cariogenic bacteria are antigenic and so initiate an immune response if they penetrate beneath the epithelial tissues of their host.^{310,331-334} This fact suggested that dental caries might be prevented by vaccinating susceptible individuals with, for instance, specific antigens^{331,333-334a} or cell preparations^{309,331,335-338} derived from cariogenic bacteria. The key role that is believed to be played by strains of *Streptococcus mutans* in the cariogenic process, and its dependence upon dextran synthesis (see p. 436), prompted research to find out whether vaccination with purified dextranase preparations derived from strains of *S. mutans*^{333-334a} might be effective in arresting the development of caries by eliciting the production of anti-dextranase antibodies that would prevent dextran synthesis. Clearly, the efficacy of this approach would be dependent upon (a) the ability of an individual to maintain raised salivary levels of anti-dextranase antibodies for

- (331) (a) P. H. De Crousaz and B. Guggenheim, *Helv. Odont. Acta*, **10**, 38 (1966);
(b) B. Guggenheim, H. R. Mühlemann, B. Regolatti, and R. Schmid, in "Dental Plaque," W. D. McHugh, ed., E. and S. Livingston, Edinburgh and London, 1970, p. 287.
- (332) A. N. Zengo, I. D. Mandel, R. Goldman, and H. S. Khurana, *Arch. Oral Biol.*, **16**, 557 (1971).
- (333) (a) S. J. Challacombe, T. Lehner, and B. Guggenheim, *Nature*, **238**, 219 (1972); (b) S. J. Challacombe, B. Guggenheim, and T. Lehner, *Arch. Oral Biol.*, **18**, 657 (1973).
- (334) J. A. Hayashi, I. L. Shklair, and A. N. Bahn, *J. Dent. Res.*, **51**, 436 (1972).
- (334a) A. Gaffer, H. W. Marcussen, H. J. Schlissel, and A. R. Volpe, *Abstr. Papers Int. Ass. Dent. Res.*, **49**, 349 (1971).
- (335) J. Carlsson and B. Krasse, *Arch. Oral Biol.*, **13**, 849 (1968).
- (336) A. E. Kennedy, I. L. Shklair, J. A. Hayashi, and A. N. Bahn, *Arch. Oral Biol.*, **13**, 1275 (1968).
- (337) T. Lehner, J. M. A. Wilton, and R. G. Ward, *Arch. Oral Biol.*, **15**, 481 (1970).
- (337a) J. M. Tanzer, G. J. Hageage, and R. H. Larson, *Arch. Oral Biol.*, **18**, 1425 (1973).
- (338) (a) W. H. Bowen, *Brit. Dent. J.*, **126**, 159 (1969); (b) **131**, 266 (1971).

prolonged periods of time, and (b) the effectiveness of these antibodies in blocking dextran synthesis. With respect to these points, the results of preliminary experiments (see Refs. 309, 331-338) have been inconclusive. However, rabbits immunized with whole streptococci^{309,335} or dextransucrase^{333b} have been found to produce antidextransucrase antibodies that inhibited dextran synthesis. Furthermore, in one group of human subjects challenged with dextransucrase preparations,³³³ the highest levels of anti-dextransucrase antibodies were, significantly, found in individuals with the least experience of oral disease, suggesting that natural exposure to *S. mutans* antigens leads to partial immunization in some individuals.

2. Dextran in the Sugar Industry

The growth of *Lactobacilli* and *Leuconostoc* micro-organisms is now generally accepted to be the most important factor contributing to the post-harvest deterioration of cane sugar³³⁹⁻³⁴³ and frost-damaged beet-sugar.³⁴⁴⁻³⁴⁸ Apart from causing significant losses of sucrose, and souring the cane or beet with organic acids, these bacteria are able to convert sucrose into dextrans that can seriously lessen the efficiency with which the deteriorated cane or beet may be processed. The adverse effects that dextrans have on the economics of sugar processing stem from a combination of several factors that together serve to lower factory and refinery capacity. Insoluble dextrans, for instance, contribute to the choking of filters, pipelines, strainers, and tanks,^{349,350} and may seriously interfere

- (339) A. (R.) Jeanes, W. C. Haynes, and C. A. Wilham, *J. Bacteriol.*, **71**, 167 (1956).
- (340) B. T. Egan and C. A. Rehbein, *Proc. Queensland Soc., Sugar Cane Technol.*, **30**, 11 (1963).
- (341) (a) B. T. Egan, *Proc. Int. Soc. Sugar Cane Technol.*, **12**, 1199 (1965); (b) **13**, 1729 (1968).
- (342) R. H. Tilbury, *Proc. Meet. West Indies Sugar Technol.*, 126 (1969).
- (343) M. E. Sharpe, E. I. Garvie, and R. H. Tilbury, *Appl. Microbiol.*, **23**, 389 (1972).
- (344) A. Vavra and I. Vavra, *Z. Zuckerind.*, **14**, 315 (1964).
- (345) A. Atterson, A. Carruthers, J. V. Dutton, D. Hibbert, J. F. T. Oldfield, M. Shore, and H. J. Teague, *Z. Zuckerind.*, **14**, 466 (1964).
- (346) A. Ya. Zagorul'ko, A. K. Buryma, S. A. Bogdanov, and T. P. Khvalkovskii, *Sakh. Prom.*, **39**, 833 (1965); *Chem. Abstr.*, **64**, 5281 (1966).
- (347) E. Schneider, H. P. Hoffmann-Walbeck, and M. A. F.-Abdou, *Zucker*, **21**, 652 (1968).
- (348) F. Schneider, E. Reinefeld, and K. Thielecke, *Zucker*, **24**, 153 (1971).
- (349) R. Moroz, in "Principles of Sugar Technology," P. Honig, ed., Elsevier Publishing Company, Amsterdam, London, and New York, 1963, Vol. 3, p. 373.
- (350) W. L. Owen, "The Microbiology of Sugars, Syrups and Molasses," Barr-Owen Research Enterprises, Louisiana (1949).

with the removal of suspended matter at the clarification stage of raw sugar manufacture.³⁵¹⁻³⁵⁷ These gums also encourage the formation of scale that results in heat losses in the sucrose juice evaporators.

The viscosities of raw sucrose juices, syrups, and massecuites (partly crystalline syrups) are increased by the presence of soluble dextrans; these have undesirable effects on the filtration, clarification, boiling, and affination processes.³⁵³⁻³⁶³ In addition, it has been demonstrated that soluble dextrans retard the rate of crystallization of sucrose, and also adversely affect the crystal shape.^{351,363-369} Normal sucrose crystals are monoclinic and slightly elongated along the b-axis, whereas crystals grown in the presence of dextran are elongated along their c-axis. The mechanism of c-axis elongation is uncertain, although it has been suggested that it is caused by the temporary adsorption of polysaccharide molecules onto certain of the growing faces of the crystal.³⁷⁰ The effect appears to be enhanced by increasing the temperature, and the molecular weight or concentration of the dextran.³⁶⁸ The formation of needle-shaped crystals is particularly undesirable, as they cause the refining quality of the sucrose to be lowered, and lessen the efficiency with which the massecuites may be purged of sucrose crystals in the centrifugal separators. A further effect of dextrans on the processing of sugar is that

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- (353) K. Vukov, *Z. Zuckerind.*, **8**, 14 (1958).
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- (361) K. Douwes-Dekker, *Proc. Sugar Ind. Technol.*, **23**, 159 (1964).
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- (363) R. H. Tilbury, *Proc. Int. Soc. Sugar Cane Technol.*, **14**, 1444 (1971).
- (364) G. Mantovani, F. Fagioli, and C. Accorsi, *Zucker*, **21**, 70 (1968).
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- (366) G. J. Leonard and G. N. Richards, *Int. Sugar J.*, **71**, 263 (1969).
- (367) (a) D. H. Foster, *Aust. Sugar J.*, **60**, 529 (1969); (b) *Proc. Queensland Soc. Sugar Cane Technol.*, **36**, 21 (1969).
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- (369) T. H. Shah and H. J. Delavier, *Z. Zuckerind.*, **24**, 27 (1974).
- (370) H. Powers, *Sugar Technol. Rev.*, **1**, 85 (1970).

they interfere with the polarimetric methods used in process control to determine sucrose and purity.³⁷¹⁻³⁷³ Dextrans are highly dextrorotatory and, unless they are removed prior to test, cause the purity of samples to be overestimated by "inflating" the direct polarization (*pol*) readouts.

From the commercial viewpoint, there is no entirely satisfactory means of preventing the microbial synthesis of dextrans in damaged cane or beet, nor of completely eliminating dextrans from deteriorated, raw sucrose juices. At the present time, the most effective way of preventing the formation of an excessive proportion of dextran in sugar cane is to minimize the delays that occur between the cutting and milling of the crop. Some diminution in the levels of dextran in raw sucrose juices is apparently achieved at the clarification stage of sugar manufacture^{354,372,374} (in which calcium oxide or some other precipitant is added to the juice^{355,360,375,376}), and consideration is now being given to the possibility of treating raw juices with preparations of dextran-degrading enzymes.^{342,363,377} A dextranase elaborated by *Penicillium funiculosum* (NRRL 1132) has been reported to lower the levels of dextran in deteriorated mill-juices, with beneficial effects upon the processing rate.³⁴² This enzyme, however, suffers from two disadvantages: it has a low thermal stability and cannot, therefore, be added at the evaporation stage of processing (which would greatly lessen treatment costs); nor is it able to degrade highly branched dextrans.^{49,140}

These and other aspects of the problems associated with the formation of dextrans in cane sugar have been excellently reviewed in an article by Imrie and Tilbury,³⁷⁸ and Cross³⁷² and D. H. Foster³⁶⁷ have, respectively, detailed the problems associated with the processing of frost-damaged and mechanically harvested cane. For further information on these subjects, the reader is referred to these articles and to the many references contained therein.

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BIBLIOGRAPHY OF CRYSTAL STRUCTURES OF CARBOHYDRATES,* NUCLEOSIDES, AND NUCLEOTIDES** 1970-1972

BY GEORGE A. JEFFREY AND MUTTAIYA SUNDARALINGAM

*Department of Crystallography, University of Pittsburgh, Pittsburgh,
Pennsylvania 15260; Department of Biochemistry, University of
Wisconsin, Madison, Wisconsin 53706*

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I. INTRODUCTION

In Volume 19 of this Series, an article¹ on crystal-structure analysis in carbohydrate chemistry detailed what was known on the subject up to *ca.* 1963; subsequent developments, up to 1970, were surveyed² in Volume 25. Since 1965, the field of X-ray crystal-structure analysis has undergone a rapid evolution because of major advances in methodology. We have now passed a transition between the era of the crystallographer who determined a structure for its own sake, and that of the newer generation of crystallographers concerned with the broader implications of a coordinated plan of attack involving groups of related molecules. In this application, crystallography provides the instrument for the study of fine detail of the molecular structure of carbohydrates in relation to their conformations and biological roles.

An up-to-date account of the theory and techniques of X-ray methods

* Work supported by NIH Grant GM-11293. The first author is grateful to Miss B. Blackmond for checking atomic parameters by use of programs written by R. Shiono.

** Work supported by NIH Grant GM-17378.

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for the determination of crystal structures has been written,³ specifically in relation to components of nucleic acids, but the principles involved are of general applicability to most organic molecules of comparable size.

Because of automation (computing, and the development⁴ of "direct methods"), crystal-structure determination has become a more routine laboratory tool for the majority of compounds with less than fifty carbon, nitrogen, and oxygen atoms, provided, of course, that suitable single crystals can be obtained. For this reason, it was decided that an annual bibliography which summarizes the *results* of application of this method to carbohydrates, nucleosides, and nucleotides and their derivatives would now be of value to carbohydrate chemists. The crystallography of the polysaccharides presents different problems and will not be included; this subject was discussed⁵ in Volume 22 of this Series. A bibliography of the structures of 92 carbohydrates and 42 nucleosides and nucleotides studied between 1935 and 1970 has been published.⁶

In the following bibliography, covering the results published in 1970–1972, the first name for the compound is that used by the authors, provided that it is acceptable; if it is not, it has been suitably modified. If that name is an accepted trivial name, the systematic name is also given. The melting point is reported whenever it is given in the crystal-structure paper. The space group and calculated density are reported, to characterize the crystalline phase of the compound which was studied. Only three-dimensional analyses are reported, as two-dimensional analyses are unreliable by present-day standards. Preliminary communications that do not include the atomic coordinates, and therefore are of more limited value, are listed separately (see Section IV, p. 464). The X-ray or neutron diffraction intensities were measured with an automatic X-ray diffractometer, unless otherwise stated. The R value or agreement index, namely, $(|F_{hkl}^{obs}| - |F_{hkl}^{calc}|)/|F_{hkl}^{obs}|$, is a "rule of thumb" commonly used as a measure of the quality of the structure analysis. The lower the value of R, the better the agreement with the experimental data. Most publications

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also give standard deviations (σ), calculated from the final stage of a least-squares refinement; these values are generally considered to be an underestimate of the errors by a factor of at least two, due to uncorrected errors of a systematic nature.

A brief comment concerning the conformation of the molecules is generally included, with use of the symbols, with sub- or super-scripts, described in the British-American Rules for Conformational Nomenclature.⁷ The torsion angles of the linkage bonds in di- and tri-saccharides are given with reference to the adjacent oxygen or carbon atoms (Cahn-Ingold-Prelog priority⁸); this procedure avoids the use of the hydrogen atoms, whose positions are poorly determined by X-ray methods, and is independent of the conformation. In the nucleosides and nucleotides, the glycosyl torsion-angles are described by the sequence O-4', C-1', N-x, C-y where $x = 1$, $y = 6$ for pyrimidines, and $x = 9$, $y = 8$ for purines. The torsion angle of the exocyclic C-4'-C-5' bond is with reference to the oxygen atoms O-4' and O-5'. The torsion angles of the nucleotide P-O ester bonds are for the sequence P-O-3', P-O-5'. Interatomic distances are given in Angström units and picometers (1 Å = 100 pm).

II. DATA FOR CARBOHYDRATES

$C_3H_5CaO_7P \cdot 3H_2O$ Calcium D-phosphoglycerate, trihydrate;⁹ calcium 3-O-phosphono-D-glycerate, trihydrate

P2₁; Z = 2; D_x = 2.238; R = 0.068 for 916 intensities (film measurements). The C(OH)CO₂⁻ part of the phosphonoglycerate ion is planar. The Ca ion is seven-coordinated by oxygen atoms in a pentagonal bipyramid. All hydroxylic hydrogen atoms form hydrogen bonds.

$C_5H_{10}O_5$ α-D-Xylopyranose¹⁰

P2₁2₁2₁; Z = 4; D_x = 1.510; R = 0.075 for 800 intensities (film measurements). The sugar is a pyranose that has the ¹C₄ conformation. The anomeric C-O bond is short. All hydroxyl hydrogen atoms form intermolecular hydrogen-bonds, including one to the ring-oxygen atom.

$C_6H_8O_4$ 1,6:2,3-Dianhydro-β-D-gulopyranose¹¹

P2₁2₁2₁; Z = 8; D_x = 1.538; R = 0.031 for 1,233 intensities. The pyranose has a half-chair conformation, ^oH₅, and the anhydro rings

(7) *J. Chem. Soc. Chem. Commun.*, 505-508 (1973).

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(9) A. Mostad and E. Rosenquist, *Acta Chem. Scand.*, **25**, 147-157 (1971).

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(11) B. Berking and N. C. Seeman, *Acta Crystallogr.*, **B27**, 1752-1760 (1971).

have the E_o and 1T_o conformations. The symmetry-independent molecules are linked by two separate, intermolecular, hydrogen-bonding systems.

$C_6H_8O_6$ Isoascorbic acid;¹² *D-arabino*-ascorbic acid

$P2_1$; $Z = 2$; $D_x = 1.668$; $R = 0.037$ for 609 intensities. The furanoid ring is planar within 0.002 Å (0.2 pm). The shape of the side chain is such as to minimize the 1,3 "peri" interactions.

$C_6H_{10}O_5$ 1,6-Anhydro- β -*D*-glucopyranose; levoglucosan,¹³ m.p. 184°C

$P2_12_12_1$; $Z = 4$; $D_x = 1.608$; $R = 0.035$ for 643 intensities. The pyranose ring, approximately 1C_4 , is distorted by the anhydro bridge, E^o , which forms a boat-shaped, seven-membered ring. There is no intramolecular hydrogen-bond, and the repulsion of the *syn*-axial hydroxyl groups also distorts the pyranose ring.

$C_6H_{10}O_5$ 1,6-Anhydro- β -*D*-mannofuranose¹⁴

$P2_12_12_1$; $Z = 4$; $D_x = 1.625$; $R = 0.039$ for 602 intensities. The *D*-mannofuranose ring is E_o , and the six-membered anhydro ring is 1C_5 .

$C_6H_{10}O_6$ *D*-Glucono-1,5-lactone¹⁵

$P2_12_12_1$; $Z = 4$; $D_x = 1.62$; $R = 0.095$ for 1,851 intensities. The pyranose ring is in a distorted, half-chair conformation, approximately 3H_4 , due to the planarity of the carbonyl group.

$C_6H_{10}O_6$ *D*-Gulono-1,4-lactone¹⁶

$P2_12_12_1$; $Z = 4$; $D_x = 1.657$; $R = 0.039$ for 800 intensities. The lactone group is planar, and the five-membered lactone ring has the E_3 conformation.

$C_6H_{11}KO_7 \cdot H_2O$ Potassium *D*-gluconate monohydrate,¹⁷ m.p. 176–180°C

$P2_12_12_1$; $Z = 4$; $D_x = 1.701$; $R = 0.061$ for 1,677 intensities. The anions have the planar, extended zigzag, carbon-chain conformation, with an

(12) N. Azarnia, H. M. Berman, and R. D. Rosenstein, *Acta Crystallogr.*, **B28**, 2157–2161 (1972).

(13) Y. J. Park, H. S. Kim, and G. A. Jeffrey, *Acta Crystallogr.*, **B27**, 220–227 (1971).

(14) J. Lechat and G. A. Jeffrey, *Acta Crystallogr.*, **B28**, 3410–3415 (1972).

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intramolecular hydrogen-bond between the *syn*-axial hydroxyl groups. The absolute configuration was confirmed. The cations have eight-fold, nearest-neighbor coordination, which does not include the carboxylate oxygen atoms.

$C_6H_{12}Br_2O_4$ 1,6-Dibromo-1,6-dideoxygalactitol¹⁸

$P2_1/c$; $Z = 2$; $D_x = 2.03$; $R = 0.131$ for 639 intensities (film measurements). The molecules are centrosymmetrical, $\bar{1}$, (C_i), in the planar, extended zigzag, carbon-chain conformation.

$C_6H_{12}Cl_2O_4$ 1,6-Dichloro-1,6-dideoxygalactitol¹⁸

$P2_1/c$; $Z = 2$; $D_x = 1.54$; $R = 0.110$ for 946 intensities (film measurements). The molecules are centrosymmetrical, $\bar{1}$, (C_i), in the planar, extended zigzag, carbon-chain conformation.

$C_6H_{12}O_4S$ (2*R*,4*S*,6*S*)-2-(Hydroxymethyl)-6-methoxy-1,4-oxathiane S-oxide,¹⁹ m.p. 95–96°C

$P2_12_12_1$; $Z = 4$; $D_x = 1.46$; $R = 0.105$ for 961 intensities (film measurements). Compound obtained by glycol cleavage of methyl 6-*O*-trityl- α -D-glucopyranoside, followed by reduction, sulfonylation, and ring-closure by sulfide anion. The 1,4-oxathiane ring has a distorted chair conformation, 5C_2 , with the sulfoxide bond equatorial and the bond to the methoxyl group axial.

$C_6H_{12}O_5 \cdot H_2O$ α -L-Rhamnopyranose monohydrate; 6-deoxy- α -L-mannopyranose²⁰

$P2_1$; $Z = 2$; $D_x = 1.457$; $R = 0.039$ for 833 intensities. Refinement of a previous analysis.^{20a} The sugar has the 1C_4 conformation.

$C_6H_{12}O_6$ *epi*-Inositol²¹

$P2_1/c$; $Z = 4$; $D_x = 1.660$; $R = 0.049$ for 1,234 intensities. There is no intramolecular hydrogen-bond between the *syn*-axial, *cis*-hydroxyl groups; instead, the oxygen atoms are forced apart, thereby introducing strain in the cyclohexane ring.

(18) K. Simon and K. Sasvari, *Acta Crystallogr.*, **B27**, 806–815 (1971).

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C₆H₁₂O₆ · CH₄N₂O α-D-Glucopyranose · urea²²

P₂₁2₁2₁; Z = 4; D_x = 1.526; R = 0.041 for 1,140 intensities. Association is by hydrogen bonds involving all of the oxygen and nitrogen atoms. The molecules are the same as in crystals of the pure, individual compounds, except for the orientation of the primary alcohol group of the D-glucose.

C₆H₁₄N⁺O₅Cl⁻ 2-Amino-2-deoxy-β-D-galactopyranose hydrochloride²³

P₂₁2₁2₁; Z = 8; D_x = 1.583; R = 0.045 for 1,248 intensities. The conformation is ⁴C₁. The C–O anomeric bond is short in both of the symmetry-independent molecules.

C₆H₁₄O₆ Allitol,^{24,25} m.p. 150–151°C

P₂₁/c; Z = 2; D_x = 1.472; R = 0.004 for 587 intensities;²⁴ R = 0.190 for 612 intensities.²⁵ The molecules are centrosymmetrical, $\bar{1}$, (C₁), and have a sickle, carbon-chain conformation.

C₆H₁₄O₆ D-Glucitol (A-form),²⁶ m.p. 85°C

P₂₁2₁2₁; Z = 4; D_x = 1.541; R = 0.032 for 797 intensities; R = 0.066 for 1,032 neutron intensities. The molecules have a sickle, carbon-chain conformation. They are linked by two hydrogen-bond spirals, one of which has O–H separations longer, and the other shorter, than normal (1.8 Å; 180 pm).

C₆H₁₄O₆ D-Iditol,²⁴ m.p. 73.5–75.0°C

P₂₁; Z = 2, D_x = 1.510; R = 0.08 for 724 intensities. The molecules have a sickle, carbon-chain conformation.

C₆H₁₄O₆ · C₅H₅N D-Glucitol · pyridine,²⁷ m.p. 76°C

P₂₁; Z = 2; D_x = 1.359; R = 0.042 for 1,161 intensities. The D-glucitol molecules have a sickle carbon-chain conformation as in D-glucitol (A-form). Layers of hydrogen-bonded D-glucitol molecules are sepa-

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rated by layers of pyridine molecules stacked in a herring-bone arrangement.

$C_7H_{12}O_5$ Methyl 3,6-anhydro- α -D-galactopyranoside²⁸

$P_{21}2_12_1$; $Z = 4$; $D_x = 1.482$; $R = 0.097$ for 1,926 intensities. The pyranose ring is distorted 1C_4 , and the anhydro ring is E^4 . The anomeric C–O bond is shortened.

$C_7H_{14}O_6$ Methyl α -D-altropyranoside²⁹

$P_{21}2_12_1$; $Z = 4$; $D_x = 1.417$; $R = 0.039$ for $\sim 1,800$ intensities. The conformation is approximately 4C_1 , with distortion caused by repulsion between the *syn*-axial hydroxyl groups. There is evidence for a weak hydrogen-bond between vicinal hydroxyl groups.

$C_7H_{14}O_6$ Methyl α -D-mannopyranoside³⁰

$P_{21}2_12_1$; $Z = 4$; $D_x = 1.463$; $R = 0.044$ for ~ 880 intensities. The molecule has the 4C_1 conformation. The anomeric C–O bond is shortened in comparison with that of α -D-mannopyranose.

$C_7H_{14}O_6 \cdot H_2O$ Methyl α -D-galactopyranoside monohydrate,³¹ m.p. $116^\circ C$

$P_{21}2_12_1$; $Z = 4$; $D_x = 1.448$; $R = 0.076$ for $\sim 1,000$ intensities. The conformation is 4C_1 . Hydrogen-bonding around the water molecules is approximately square planar.

$C_7H_{14}O_7$ Coriose; D-altro-3-heptulose,³² m.p. 169 – $171^\circ C$

P_{21} ; $Z = 2$; $D_x = 1.584$; $R = 0.080$ for 1,046 intensities (film measurements). The molecule has the α -D configuration and a furanose ring, 5T_6 , with the three hydroxyl groups *cis* to each other, and *trans* to the primary and secondary alcohol groups.

$C_8H_{16}O_6S$ Methyl 6-deoxy-6-(methylsulfinyl)- α -D-glucopyranoside³³

$P3_2$; $Z = 3$; $D_x = 1.46$; $R = 0.100$ for 356 intensities (film measurements). The absolute configuration at the sulfur atom is S, in agreement with the o.r.d. data.

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(31) B. M. Gatehouse and B. J. Poppleton, *Acta Crystallogr.*, **B27**, 654–660 (1971).

(32) T. Taga, K. Osaki, and T. Okuda, *Acta Crystallogr.*, **B26**, 991–997 (1970).

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$C_9H_{14}IN_3O_4$ 6-Azido-5,6-dideoxy-5-iodo-1,2-*O*-isopropylidene- β -L-ido-furanose³⁴

$P2_1$; $Z = 2$; $D_x = 1.83$; $R = 0.197$ for 830 intensities (film measurements). The fused furanose and dioxolane rings have E_4 and E_7 conformations respectively, and the C-4-C-5 bond is *quasi*-equatorial.

$C_{10}H_{16}KNO_9S_2 \cdot H_2O$ Sinigrin monohydrate (potassium salt);³⁵ potassium myronate

$P2_12_12_1$; $Z = 4$; $D_x = 1.644$; $R = 0.056$ for 2,045 intensities. The conformation of the β -D-glucosyl group is 4C_1 . The absolute configuration at C-1 was confirmed.

$C_{10}H_{20}O_4S_2$ Ethyl 2-*S*-ethyl-1,2-dithio- α -D-mannofuranoside³⁶

$P2_12_12_1$; $Z = 4$; $D_x = 1.34$; $R = 0.110$ for 552 intensities (film measurements). The two *S*-alkyl groups are *trans*, with the *manno* configuration. The conformation lies between E_4 and 3T_4 .

$C_{10}H_{22}N^+O_4I^-$ 4,6-Dideoxy-4-(dimethylamino)- α -D-talopyranoside methiodide³⁷

$P2_12_12_1$; $Z = 4$; $D_x = 1.690$; $R = 0.041$ for 798 intensities. The conformation is 1C_4 , due to the presence of the NMe_2 group at C-4.

$C_{11}H_{17}NO_5$ 3,6-(Acetylepimino)-3,6-dideoxy-1,2-*O*-isopropylidene- β -L-idofuranose,³⁸ m.p. 169–170°C

$P2_1$; $Z = 2$; $D_x = 1.258$; $R = 0.078$ for 946 intensities. All three five-membered rings have an envelope shape; the furanose ring is E_4 . The dihedral angle between the planes is 114°.

$C_{12}H_{12}O_{12}$ Dehydro-L-ascorbic acid dimer,³⁹ m.p. 225–235°C (dec.)

$C2$; $Z = 2$; $D_x = 1.836$; $R = 0.061$ for 730 intensities (film measurements). The molecule has 2-fold axial symmetry, (C_2), with two 1,4-lactone and two furanose rings attached to a central 1,4-dioxane ring. The 1,4-dioxane ring has the 0S_2 conformation. The lactone rings are E_3 and the furanose ring is 3T_0 .

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$C_{12}H_{16}O_8$ 3,6-Anhydro- α -D-glucopyranosyl 1,4:3,6-dianhydro- β -D-fructofuranoside⁴⁰

$P2_12_12_1$; $Z = 4$; $D_x = 1.57$; $R = 0.071$ for 1,161 intensities. The pyranose is 1C_4 and the furanose is 4T_3 . The anhydro rings are E^4 . The linkage torsion-angles are $+84^\circ$, $+69^\circ$ (Glc \rightarrow Fru). The hydrogen bonding is all intermolecular.

$C_{12}H_{22}O_{11} \cdot H_2O$ α -Lactose monohydrate; 4-O- β -D-galactopyranosyl- α -D-glucopyranose monohydrate^{41,42}

$P2_1$; $Z = 2$; $D_x = 1.524$; $R = 0.027$ for 1,296 intensities;⁴¹ $R = 0.150$ for 1,379 intensities (film measurements).⁴² Both of the pyranose conformations are 4C_1 ; the linkage torsion-angles are -94° , $+96^\circ$ (Gal \rightarrow Glc). The crystal examined contained 7 percent random substitution by the β -D anomer. There is an intramolecular hydrogen-bond between O-3 of the D-galactosyl group and the ring-oxygen atom of the D-glucose residue.

$C_{12}H_{22}O_{11} \cdot H_2O$ β -Maltose monohydrate; 4-O- α -D-glucopyranosyl- β -D-glucopyranose monohydrate⁴³

$P2_1$; $Z = 2$; $D_x = 1.532$; $R = 0.103$ for 781 intensities (film measurements). The D-glucosyl group and the D-glucose residue have the 4C_1 conformation. The linkage torsion angles are 123° , 132° . There is an intramolecular hydrogen-bond between O-2 and O-3 of the two residues.

$C_{12}H_{22}O_{11} \cdot 2H_2O$ α,α -Trehalose dihydrate; α -D-glucopyranosyl α -D-glucopyranoside dihydrate,^{44,45} m.p. $96-97^\circ C$

$P2_12_12_1$; $Z = 4$; $D_x = 1.511$; four independent determinations; $R = 0.057$ for 3,293 intensities, $R = 0.041$ for 1,611 intensities, and $R = 0.055$ for 2,149 intensities;⁴⁴ $R = 0.057$ for 1,892 intensities (film measurements).⁴⁵ The molecule has approximate 2-fold symmetry (C_2). The conformations of the D-glucosyl groups are 4C_1 . The linkage torsion-angles are $+62^\circ$, $+75^\circ$.

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$C_{13}H_{24}O_{11} \cdot CH_3OH$ Methyl β -cellobioside methanolate; methyl 4-*O*- β -D-glucopyranosyl- β -D-glucopyranoside methanolate⁴⁶

$P2_1$; $Z = 2$; $D_x = 1.453$; $R = 0.063$ for 1,724 intensities (film measurements). The conformations are 4C_1 ; the linkage bonds are $+91^\circ$, -80° . There is evidence of a bifurcated, intramolecular hydrogen-bond from O-3' of the D-glucoside residue to the ring-oxygen atom (O-5) and the primary alcoholic oxygen atom (O-6) of the D-glucosyl group.

$C_{14}H_{16}O_5$ Methyl 2,3-anhydro-4,6-*O*-benzylidene- α -D-mannopyranoside⁴⁷

$P2_1$; $Z = 2$; $D_x = 1.659$; $R = 0.039$ for 1,537 intensities. The molecule has the 4C_1 conformation, flattened due to the epoxide ring; the acetal ring also has a distorted-chair shape.

$C_{14}H_{24}O_6S$ Ethyl 2,3:4,5-di-*O*-isopropylidene-1-thio- β -D-glucoseptanoside⁴⁸

$P2_12_12_1$; $Z = 4$; $D_x = 1.18$; $R = 0.103$ for 1,322 intensities (film measurements). The septanose ring is a twist-chair (2-fold axis through C-1) and the dioxolane rings are ${}^0\text{-}^3T_{C-3}$ and ${}^C\text{-}^5T_{O-5}$.

$C_{17}H_{26}BrNO_5$ *N*-(*p*-Bromobenzyl)nogalonamide; *N*-(*p*-bromobenzyl)-6-deoxy-3-*C*-methyl-2,3,4-tri-*O*-methyl-L-mannonamide,⁴⁹ m.p. 120–121°C

$P2_1$; $Z = 2$; $D_x = 1.384$; $R = 0.071$ for 3,427 intensities. Absolute configuration confirms that nogalose is a derivative of 6-deoxy-L-mannose. The molecule has an open-chain shape, with a sickle carbon-chain conformation, thereby permitting formation of an intramolecular hydrogen-bond between O-1-H and O-5-H at either end of the chain.

$C_{18}H_{21}BrO_7S$ 5-*O*-(*p*-Bromophenylsulfonyl)-2,2'-*O*-cyclohexylidene-3-deoxy-2-*C*-(hydroxymethyl)-D-*erythro*-pentono-1,4-lactone⁵⁰

$P2_1$; $Z = 2$; $D_x = 1.59$; $R = 0.100$ for 1,140 intensities. Both five-membered rings have an *E* conformation; the 1,3-dioxolane ring has greater displacement than the 1,4-lactone ring. The cyclohexylidene ring has the 4C_1 conformation. The structure determination provided

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confirmation of the absolute configuration of " α "-D-isosaccharinic acid [3-deoxy-2-C-(hydroxymethyl)-D-*erythro*-pentonic acid].

$C_{18}H_{32}O_{16}$ 1-Kestose; O- β -D-fructofuranosyl-(2 \rightarrow 1)-O- β -D-fructofuranosyl α -D-glucopyranoside,⁵¹ m.p. $\sim 184^\circ\text{C}$

$P2_12_12_1$; $Z = 4$; $D_x = 1.582$; $R = 0.039$ for 2,089 reflections. The conformation of the D-glucosyl group is 4C_1 ; those of the D-fructose residues are ${}^3T^4$ and E_3 . The linkage torsion-angles are 85° , -66° (Glc \rightarrow Fru); 179° , -170° , -41° (Fru \rightarrow Fru). There is no intramolecular hydrogen-bond.

$C_{18}H_{32}O_{16} \cdot 2H_2O$ Planteose dihydrate; O- α -D-galactopyranosyl-(1 \rightarrow 6)-O- β -D-fructofuranosyl α -D-glucopyranoside dihydrate,⁵² m.p. 123°C

$P2_12_12_1$; $Z = 4$; $D_x = 1.531$; $R = 0.036$ for 2,197 intensities. The conformations of the pyranose moieties are 4C_1 and that of the furanosyl residue is 4T_3 . The linkage torsion-angles are 59° , 173° , 64° (Gal \rightarrow Fru); -27° , 109° (Fru \rightarrow Glc). There is no intramolecular hydrogen-bond.

$C_{18}H_{32}O_{16} \cdot 5H_2O$ Raffinose pentahydrate; O- α -D-galactopyranosyl-(1 \rightarrow 6)-O- α -D-glucopyranosyl β -D-fructofuranoside pentahydrate,⁵³ m.p. 118°C

$P2_12_12_1$; $Z = 4$; $D_x = 1.496$; $R = 0.060$ for 2,097 intensities. The conformations of the pyranose residues are 4C_1 , and that of the furanoside is 4T_3 . The linkage torsion-angles are 72° , -170° , -65° (Gal \rightarrow Glc), 82° , 11° (Glc \rightarrow Fru). There is no intramolecular hydrogen-bond.

$C_{21}H_{20}BrN_7O_7S$ Methyl 4,6-O-benzylidene-2-O-(*p*-bromophenylsulfonyl)-3-cyano-3-deoxy- α -D-altropyranoside⁵⁴

$P1$; $Z = 1$; $D_x = 1.572$; $R = 0.122$ for 1,810 intensities (film measurements). The conformation of the pyranose is approximately 4C_1 with distortion due to *syn*-axial repulsion of the substituents at C-1 and C-3. The acetal ring is in a chair conformation, with the phenyl group equatorially attached.

$C_{26}H_{32}O_{10}S$ 1,2:5,6-Di-O-isopropylidene-3,4-di-O-*p*-tolylsulfonyl-L-*chiro*-inositol⁵⁵

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P₄2₁2; Z = 4; D_x = 1.35; R = 0.04 for 1,617 intensities. The molecule has a 2-fold axis of symmetry, C₂. The cyclohexane ring is flattened, ¹S₃, with oxygen atoms 1, 3, 4, and 6 axial; the dioxolane rings are ⁷T₀₋₁.

III. DATA FOR NUCLEOSIDES AND NUCLEOTIDES

C₉H₁₀N₂O₇PS⁻ · C₆H₁₅N⁺ Uridine 2',3'-phosphorothioate, triethylammonium salt,⁵⁶ m.p. 204–205°C

P₂2₁2₁; Z = 4; D_x = 1.408; R = 0.096 for 986 intensities. The conformation of the D-ribosyl moiety is ⁶T^{*}, the glycosyl disposition is *anti* (–13°), and the exocyclic, C-4'–C-5' bond torsion-angle is 168°. The cyclic phosphoric diester ring is puckered, with the phosphorus atom displaced 0.21 Å (21 pm) out of the remaining, four-atom plane. The dihedral angle between the latter plane and the C-1'–C-2'–C-3'–C-4' plane of the D-ribosyl residue is 66°. There is an inter-base hydrogen-bond between N-3–H and the carbonyl oxygen atom (O-4).

C₉H₁₁ClN₂O₅ 2'-Chloro-2'-deoxyuridine⁵⁷

P₂2₁2₁; Z = 4; D_x = 1.574; R = 0.036 for 1,379 intensities. The conformation of the substituted D-ribosyl group is ²T₁, the glycosyl disposition is *anti* (62°), and the C-4'–C-5' torsion-angle is –70°.

C₉H₁₁ClN₂O₆ 5-Chlorouridine⁵⁸

P₂₁; Z = 2; D_x = 1.627; R = 0.032 for 1,061 intensities. The conformation of the D-ribosyl group is ²T₃, the glycosyl disposition is *anti* (51°), and the exocyclic C-4'–C-5' bond torsion-angle is –66.3°. Inter-base hydrogen-bonds between N-3–H and the carbonyl oxygen atom (O-4) form an infinite spiral around the screw axis.

C₉H₁₁IN₂O₆ 5-Iodouridine⁵⁹

P₂₁; Z = 2; D_x = 2.102; R = 0.061 for 2,143 intensities. There are two symmetry-independent molecules; the conformation of the D-ribosyl group is ³T₂ in one molecule and ²T₃ in the other. The glycosyl dispositions are both *anti* (12°, 56°). The exocyclic, C-4'–C-5' bond torsion-angles are –63°, –177°.

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$C_9H_{11}NO_6$ Showdomycin, 3- β -D-ribofuranosyl-3-pyrroline-2,5-dione,⁶⁰
m.p. 160–161°C

$C2_1$; $Z = 4$; $D_x = 1.540$; $R = 0.050$ for 1,092 intensities. The conformation of the D-ribosyl group is 2T_1 , the glycosyl disposition is *syn* (-132°), and the exocyclic, C-4'-C-5' torsion-angle is -63° . There is an intramolecular hydrogen-bond between the carbonyl oxygen atom (O-2) in the pyrroline ring and the O-5' atom of the D-ribosyl group.

$C_9H_{11}N_2Na_2O_9P \cdot 4H_2O$ Uridine 3'-phosphate, disodium salt, tetrahydrate^{60a}

$P2_12_12_1$; $Z = 4$, $D_x = 1.686$; $R = 0.043$ for 2,426 intensities. The conformation of the D-ribosyl residue is 2T_1 , the glycosyl disposition is *anti* (45°), and the exocyclic C-4'-C-5' bond torsion-angle is 43° . There is an interbase hydrogen-bond involving the ring-nitrogen atom, N-3-H, of one base and the carbonyl oxygen atom, O-4, of a neighboring base. Atom Na-1 is octahedrally coordinated to 6 water molecules, and Na-2 is coordinated to 3 water molecules and to the hydroxyl oxygen atoms O-2', O-5' and the ester oxygen atom O-3' of neighboring molecules. The sodium octahedra share faces to form an extended, polyhedral network.

$C_9H_{12}N_2O_4S_2 \cdot H_2O$ 2,4-Dithiouridine monohydrate^{61,62}

$P2_12_12_1$; $Z = 4$; $D_x = 1.569$; two independent investigations: $R = 0.039$ for 1,297 intensities;⁶¹ $R = 0.039$ for 1,436 intensities.⁶² The conformation of the D-ribosyl group is 3T_2 , the glycosyl disposition is *anti* (19°), and the exocyclic C-4'-C-5' bond torsion-angle is -77° . There is an inter-base hydrogen-bond between N-3-H and S-4.

$C_9H_{12}N_2O_5$ 2'-Deoxyuridine⁶³

$P2_1$; $Z = 4$; $D_x = 1.611$; $R = 0.084$ for 1,611 intensities. There are two symmetry-independent molecules. The conformations of the 2-deoxy-D-*erythro*-pentofuranosyl groups are 2T_3 , and the glycosyl dispositions are *anti* (26 and 28°). The exocyclic C-4'-C-5' bond torsion-angles are 174 and 167° .

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(63) A. Rahman and H. R. Wilson, *Acta Crystallogr.*, **B28**, 2260–2270 (1972).

C₉H₁₂N₂O₅S·1.5H₂O 4-Thiouridine sesquihydrate⁶⁴

C2; Z = 4; D_x = 1.495; R = 0.053 for 1,381 intensities. The conformation of the D-ribose group is ³T₄, the glycosyl disposition is *syn* (−97°), and the exocyclic C-4′–C-5′ bond torsion-angle is −52°. The water molecules are connected by hydrogen bonds, one of which is hydrogen-bonded to the carbonyl atom (O-2) of the base, and the other to O-5′. The base rings are stacked, and the sulfur atom is not hydrogen-bonded.

C₉H₁₂N₂O₆·H₂O α-Pseudouridine monohydrate⁶⁵

P2₁; Z = 2; D_x = 1.526; R = 0.041 for 1,038 intensities. The conformation of the D-ribose group is ₂E, the glycosyl disposition is *anti* (3°), and the exocyclic C-4′–C-5′ bond torsion-angle is 70°. There is one interbase hydrogen-bond between N-1–H and the carbonyl oxygen atom (O-4).

C₉H₁₃N₃O₄S·2H₂O 2-Thiocytidine dihydrate⁶⁶

P1; Z = 1; D_x = 1.510; R = 0.039 for 1,103 intensities. The conformation of the D-ribose group is ³T₂, the glycosyl disposition is *anti* (20°), and the exocyclic C-4′–C-5′ bond torsion-angle is −64°. The sulfur atom is hydrogen-bonded to the amino group of a neighboring molecule and to the water.

C₉H₁₄ClN₃O₄ 2′-Deoxycytidine hydrochloride⁶⁷

P2₁; Z = 2; D_x = 1.551; R = 0.035 for 2,382 intensities. The conformation of the 2-deoxy-D-*erythro*-pentofuranosyl group is ³T₂, the glycosyl disposition is *anti* (0°), and the exocyclic C-4′–C-5′ bond torsion-angle is −72°. The N-3 atom of the cytosine ring is protonated.

C₉H₁₄N₂O₆·0.5H₂O Dihydrouridine hemihydrate^{68,69}

P2₁2₁2₁; Z = 8; D_x = 1.538; two independent investigations: R = 0.056 for 1,917 intensities;⁶⁸ R = 0.051 for 2,143 intensities.⁶⁹ There are

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two symmetry-independent molecules. The conformation of both *D*-ribose groups is 2T_1 and the glycosyl dispositions are *anti* (65 and 57°). The exocyclic C-4'-C-5' bond torsion-angle in molecule 1 is 51°. In molecule 2, the O-5'-H group is two-fold disordered according to one investigation, and the torsion angles are 168 and -77°; according to the other investigation, it is three-fold disordered, and the torsion angles are 162°, -77°, and -174°.

$C_9H_{14}N_3O_7P \cdot H_2O$ 2'-Deoxycytidine 5'-phosphate monohydrate⁷⁰

$P2_12_12_1$; $Z = 4$; $D_x = 1.671$; two independent investigations: $R = 0.074$ for 1,149 intensities (film measurements); $R = 0.023$ for 1,197 intensities. The conformation of the *D*-ribose group is ${}_3T_4$, the glycosyl disposition is *anti* (-6°), and the C-4'-C-5' bond torsion-angle is 63°. Atom N-3 of the cytosine ring is protonated.

$C_{10}H_{11}BrN_5O_5 \cdot 2H_2O$ 8-Bromoguanosine dihydrate⁷¹

$P2_12_12_1$; $Z = 4$; $D_x = 1.816$; $R = 0.055$ for 1,149 intensities. The conformation of the *D*-ribose group is 2T_1 , the glycosyl disposition is *syn* (-130°), and the exocyclic C-4'-C-5' torsion-angle is -65°. An intramolecular hydrogen-bond connects O-5'-H with N-3 of the guanine ring.

$C_{10}H_{12}BrN_5O_3 \cdot C_{17}H_{20}N_4O_6 \cdot 3H_2O$ 5'-Bromo-5'-deoxyadenosine-riboflavine complex, trihydrate⁷²

$P2_12_12_1$; $Z = 4$; $D_x = 1.538$ for 1,152 intensities. The conformation of the *D*-ribose group is 2T_1 , the glycosyl disposition is *syn* (-106°), and the exocyclic C-4'-C-5' bond torsion-angle is 176°. The adenine and isoalloxazine rings are associated by a pair of hydrogen bonds involving N-6-H and O-2 of the adenine and N-7 and H-N-3 of the adenine. The two rings are also alternately stacked, with their long axes approximately parallel.

$C_{10}H_{12}BrN_5O_4$ 8-Bromoadenosine⁷¹

$P2_1$; $Z = 2$; $D_x = 1.822$; $R = 0.045$ for 1,104 intensities. The conformation of the *D*-ribose group is 2T_3 , the glycosyl disposition is *syn* (-120°), and the exocyclic C-4'-C-5' bond torsion-angle is -74°. An intramolecular hydrogen-bond connects the 5-hydroxyl group with N-3 of the adenine ring.

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C₁₀H₁₂N₄O₅ Inosine⁷³

P2₁; Z = 2; D_x = 1.613; R = 0.047 for 1,334 intensities. The conformation of the D-ribose group is ³T₂, the glycosyl disposition is *anti* (12°), and the exocyclic C-4'-C-5' torsion-angle is 75°.

C₁₀H₁₂N₄O₅·2H₂O Inosine dihydrate^{74,75}

P2₁; Z = 4; D_x = 1.548; two independent determinations: R = 0.032 for 1,423 intensities;⁷⁴ R = 0.09 for 2,347 intensities.⁷⁵ There are two symmetry-independent molecules. In molecule 1, the conformation of the D-ribose group is ²T₃, the glycosyl disposition is *syn* (120°), and the exocyclic C-4'-C-5' bond torsion-angle is -55°. In molecule 2, the conformation is ²T₁, the glycosyl disposition is *anti* (49°), and the exocyclic C-4'-C-5' torsion-angle is -73°. The bases are stacked, with extensive overlap of the rings.

C₁₀H₁₃N₅O₄ Adenosine⁷⁶

P2₁; Z = 2; D_x = 1.533; R = 0.024 for 1,333 intensities. The conformation of the D-ribose group is ³T₂, the glycosyl disposition is *anti* (10°), and the exocyclic C-4'-C-5' bond torsion-angle is 60°.

2(C₁₀H₁₃N₅O₄)·C₆₂H₈₄N₁₂O₁₆·12H₂O 2 (2'-Deoxyguanosine)-actinomycin complex, dodecahydrate⁷⁷

P2₁2₁2₁; Z = 8; D_x = 1.340; R = 0.094 for 4,000 intensities. There are two 2'-deoxyguanosine molecules stacked on each side of the actinomycin chromophore; they are related to each other by an approximate diad passing through the N-O bridging atoms of the phenoxazone ring. In addition, the 2'-deoxyguanosine molecules are connected to the cyclic peptides by two hydrogen-bonds involving (a) the amino group of guanine and the carbonyl oxygen atom of the L-threonine residue, and (b) N-3 of the guanine ring and the N-H group of the same L-threonine residue. The conformation of the 2-deoxy-D-*erythro*-pentofuranosyl group in molecule 1 is ³T₂, the glycosyl disposition is *anti* (89°), and the exocyclic C-4'-C-5' bond torsion-angle is 75°. In molecule 2, the conformation is ²T₁, the glycosyl disposition is *syn* (93°), and the exocyclic C-4'-C-5' torsion-angle is 66°.

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C₁₀H₁₃N₅O₄S·H₂O 6-Thioguanosine monohydrate⁷⁸

C222₁; Z = 8; D_x = 1.532; R = 0.073 for 1,285 intensities. The conformation of the D-ribose group is ²T₁, the glycosyl disposition is *anti* (66°), and the exocyclic C-4'-C-5' bond torsion-angle is 64°. The bases are so stacked that their sulfur atoms lie in close contact with the pyrimidine rings of adjacent molecules.

C₁₀H₁₃N₅O₅·2H₂O Guanosine dihydrate⁷⁴

P2₁; Z = 4; D_x = 1.597; R = 0.036 for 2,946 intensities. There are two symmetry-independent molecules; the conformations of the D-ribose groups are ²E and ¹T², the glycosyl dispositions are respectively *syn* (123°) and *anti* (47°), and the exocyclic C-4'-C-5' bond torsion-angles are +51 and -74°, respectively.

C₁₀H₁₄N₂O₈ 6-Methyluridine⁷⁹

P2₁; Z = 4; D_x = 1.543; R = 0.049 for 2,670 intensities. There are two symmetry-independent molecules. The conformation of the D-ribose group in molecule 1 is ²T₁, and in molecule 2, it is ²T₃. The glycosyl dispositions are *syn* (-109 and -107°). The exocyclic C-4'-C-5' bond torsion-angles are -69 and +62°. Only the first molecule exhibits an intramolecular hydrogen-bond between O-5'-H and the carbonyl oxygen atom (O-2) of the base. The rings of the base of the two molecules are stacked alternately, at interplanar spacings of 3.24 Å (324 pm).

C₁₀H₁₄N₅Na₂O₁₃P₃·3H₂O Adenosine 5'-triphosphate, disodium salt, trihydrate⁸⁰

P2₁2₁2₁; Z = 8; D_x = 1.787; R = 0.123 for 1,118 intensities. There are two symmetry-independent molecules. In one molecule, the conformation of the D-ribose residue is ³T₂, the glycosyl disposition is *anti* (69°), and the exocyclic C-4'-C-5' bond torsion-angle is -48°. In the other molecule, the conformation is ²T₃, the glycosyl disposition is *anti* (39°), and the exocyclic C-4'-C-5' torsion-angle is -58°. The triphosphate chain is folded into a left-handed helix in molecule 1, and into a right-handed helix in molecule 2. Two of the sodium ions coordinate the two adenosine triphosphate molecules through the phos-

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phate oxygen atoms and N-7 of the adenine ring, to form an almost centrosymmetric "dimer" which is the fundamental structural unit.

$C_{10}H_{14}N_6O_3 \cdot H_2O$ 9-(2-Amino-2-deoxy- α -D-ribofuranosyl) adenine monohydrate⁸¹

P2₁2₁2₁; Z = 4; D_x = 1.529; R = 0.053 for 1,074 intensities. The conformation of the D-ribosyl group is ${}_3T^2$, the glycosyl disposition is *anti* (-60°), and the exocyclic C-4'-C-5' bond-angle is 53° . There is an intramolecular hydrogen-bond between the N-H of the 2-amino group and O-3'. The pyrimidine parts of adjacent bases are stacked.

$C_{10}H_{16}N_2O_5$ Dihydrothymidine⁸²

P2₁2₁2₁; Z = 4; D_x = 1.419; R = 0.051 for 1,188 intensities. The conformation of the 2-deoxy-D-*erythro*-pentofuranosyl group is oT_4 , the glycosyl conformation is *anti* (53°), the O-5-H group is two-fold disordered, and the torsion angles are -64 and $+74^\circ$. The dihydrothymine ring has a half-chair conformation in which C-5 exhibits the largest displacement from the plane of that ring.

$C_{11}H_{16}N_5O_5P \cdot C_2H_5OH$ 3'-Deoxy-3'-(dihydroxyphosphinylmethyl)-adenosine ethanolate⁸³

P2₁2₁2₁; Z = 4; D_x = 1.507; R = 0.044 for 1,417 intensities. The conformation of the D-ribosyl residue is 3T_2 , the glycosyl disposition is *anti* (28°), and the exocyclic C-4'-C-5' bond torsion-angle is -69° . Atom N-1 of the adenine ring is protonated.

$C_{11}H_{17}IN_6O_3 \cdot H_2O$ 5'-Deoxy-5'-(methylammonium)adenosine iodide, monohydrate⁸⁴

P2₁; Z = 2; D_x = 1.715; R = 0.041 for 1,415 intensities. The conformation of the D-ribosyl group is 2T , the glycosyl disposition is *syn* (-151°), and the exocyclic C-4'-C-5' bond torsion-angle is 43° . There is an intramolecular hydrogen-bond between N-5' (the ammonium nitrogen atom) and N-3 of the adenine ring. There are two iodide ions, one on each side of the adenine ring, at average distances of 3.82 Å (382 pm), suggesting interaction of the charge-transfer type between the iodide ion and the base.

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(83) S. Hecht and M. Sundaralingam, *J. Amer. Chem. Soc.*, **94**, 4314-4319 (1972).

(84) W. Saenger, *J. Amer. Chem. Soc.*, **93**, 3035-3041 (1971).

C₁₂H₁₅N₅O₅ 3'-O-Acetyladenosine⁸⁵

P₂₁2₁2₁; Z = 4; D_x = 1.504; R = 0.041 for 1,189 intensities. The conformation of the D-ribose group is ²T₁, the glycosyl disposition is *syn* (−133°), and the exocyclic C-4'-C-5' torsion-angle is −61°. The torsion angles about the acetic ester bond (C-3'-O-3') are +98 and −150°. There is an intramolecular hydrogen-bond between O-5'-H and N-3 of the base.

C₁₂H₁₅N₆O₅ N²,N²-Dimethylguanosine⁸⁶

P₂₁2₁2₁; Z = 4; D_x = 1.547; R = 0.046 for 1,125 intensities. The conformation of the D-ribose group is ²T₃, the glycosyl disposition is *syn* (−104°), and the exocyclic C-4'-C-5' bond torsion-angle is 65°. The dimethylamino groups are sandwiched by the pyrimidine rings of adjacent bases.

C₁₂H₁₆N₂O₅S 3'-O-Acetyl-4-thiothymidine⁸⁷

P₂₁2₁2₁; Z = 4; D_x = 1.434; R = 0.054 for 1,213 intensities. The conformation of the 2-deoxy-D-*erythro*-pentopyranosyl residue is ²T₃, the glycosyl disposition is *anti* (54°), and the exocyclic C-4'-C-5' bond torsion-angle is −63°. The torsion angles about C-3'-O-3' (the acetic ester bond) are +121 and −129°. The only intermolecular hydrogen-bond is between O-5'-H and the sulfur atom. The N-3-H group of thymine does not participate in hydrogen bonding.

C₁₃H₁₆N₄O₆ 6,7-Dimethyl-N¹-β-D-ribofuranosyl lumazine^{87a}

P₂; Z = 4; D_x = 1.521; R = 0.059 for 4,114 intensities. There are two symmetry-independent molecules, and both exhibit the *syn* (−99°, −106°) conformation and an intramolecular hydrogen-bond between the 5'-OH group of the D-ribose group and the carbonyl oxygen atom (O-2). The D-ribose groups are ²T₃ and ²T₁, and the exocyclic, C-4'-C-5' torsion-angles are −68° and −64°. The symmetry-independent molecules are related by a pseudo-diad. The bases of the pteridine half are stacked. The nitrogen atoms of the pyrazine half are not involved in hydrogen bonding. The lumazine bases are nonplanar, and the pyrimidine and pyrazine rings make dihedral angles of about 5°.

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C₁₅H₂₄N₇O₁₂P·0.5H₂O Uridyl-yl-(3'→5')-adenosine hemihydrate^{88,89}

P₂₁; Z = 4; D_x = 1.651; two independent determinations: R = 0.081 for 2,680 intensities;⁸⁸ R = 0.040 for 3,179 intensities.⁸⁹ There are two symmetry-independent molecules. All four nucleoside residues have similar conformations; the conformations of the D-ribose residues are ³T₂, the glycosyl dispositions are *anti** (U-1, 20°; A-1, 49°; U-2, 10°; and A-2, 36°), and the exocyclic C-4'-C-5' torsion-angles are, respectively, U-1, -63°; A-1, -68°; U-2, -68°; and A-2, -62°. The two molecules are strikingly different in the orientations of the phosphoric diester linkages. The linkage torsion-angles are: UpA-1, 163 and -88°, and UpA-2, 84 and 84°. The N-1 atoms of the adenine rings are protonated. The structure exhibits both A-A and U-U self-pairs; the former is connected by two N-6-H···N-7 hydrogen bonds; the latter, by the hydrogen bonds N-3-H···O-4 and O-2···H-N-3. The adenine nucleotide residues of the A-A pair are related by an approximate diad. The crystal contains alternating sheets of adenine and uracil residues.

C₂₂H₂₉N₇O₅·2HCl·5H₂O Puromycin dihydrochloride, pentahydrate;
9-[3-deoxy-3-(*p*-methoxyphenyl-L-alanyl-amino)-β-D-ribofuranosyl]-6-(dimethylamino)purine dihydrochloride, pentahydrate⁹⁰

P₂₁2₁2₁; Z = 4; D_x = 1.421; R = 0.055 for 1,968 intensities. The conformation of the D-ribose residue is ³T₂, the glycosyl disposition is *anti* (19°), and the exocyclic C-4'-C-5' bond torsion-angle is 65°. The torsion angle about the C-2-C-3 bond of the amino acid moiety is -169°. The torsion angles of the aminoacyl linkage about the C-3'-N-3' and N-3'-C-1 bonds are -135 and +177°. The puromycin molecule exists as a di-cation, and N-1 of the adenine ring and the amino nitrogen atom are protonated. The molecule is extended, with the aromatic ring and the base lying on opposite sides of the peptide group. There is no intramolecular ring-stacking; however, the adenine and *p*-methoxyphenyl rings of adjacent molecules are stacked alternately, at interplanar spacings of about 3.4 Å (340 pm).

IV. PRELIMINARY COMMUNICATIONS

The following is a list of compounds for which results are given in Preliminary Communications; these are of limited value, as they do not

* A = adenosine residue, and U = uridine residue.

(88) J. L. Sussman, N. C. Seeman, S.-H. Kim, and H. M. Berman, *J. Mol. Biol.*, **66**, 403-421 (1972).

(89) J. Rubin, T. Brennan, and M. Sundaralingam, *Biochemistry*, **11**, 3112-3128 (1972).

(90) M. Sundaralingam and S. K. Arora, *J. Mol. Biol.*, **71**, 49-70 (1972).

include atomic parameters, and may be superseded by full reports to appear later.

1. Carbohydrates

- $C_3H_7Na_2O_8P \cdot 6H_2O$ Disodium DL-glycerol 3-phosphate, hexahydrate^{91,92}
 $C_6H_{12}O_3S_2 \cdot 0.5H_2O$ Methyl 1,5-dithio- α -D-ribopyranoside, hemihydrate⁹³
 $C_6H_{12}O_4S$ Methyl 1-thio- α -D-ribopyranoside⁹³
 $C_6H_{12}O_6 \cdot H_2O$ α -D-Glucose monohydrate⁹⁴
 $C_6H_{12}O_6 \cdot CaBr_2 \cdot 3H_2O$ α -D-Galactopyranose·calcium bromide, trihydrate⁹⁵
 $C_6H_{12}O_6 \cdot CaBr_2 \cdot 5H_2O$ *myo*-Inositol·calcium bromide, pentahydrate⁹⁵
 $C_6H_{12}O_6 \cdot CaCl_2 \cdot 4H_2O$ β -D-Mannofuranose·calcium chloride, tetrahydrate⁹⁶
 $C_{10}H_{19}NO_8 \cdot 3H_2O$ Methoxyneuramic acid, trihydrate⁹⁷
 $C_{11}H_{13}O_5$ and $C_{18}H_{16}BrO_8$ α -D-Xylopyranose 1,2,4-orthobenzoate and its 3-(*p*-bromobenzoate)⁹⁸
 $C_{13}H_{24}O_5S$ Ethyl 2,3:4,5-di-*O*-isopropylidene-1-thio- β -D-glucoseptanoside⁹⁹
 $C_{14}H_{20}O_7$ 3-*O*-Acetyl-1,2:4,5-di-*O*-isopropylidene- α -D-glucoseptanose¹⁰⁰
 $C_{14}H_{21}ClO_7$ 5-*O*-(Chloroacetyl)-1,2:3,4-di-*O*-isopropylidene- α -D-glucoseptanose¹⁰¹
 $C_{15}H_{20}O_{12}$ Methyl 2,3,4,5-tetra-*O*-acetyl- β -D-glucoseptanoside⁹⁹
 $C_{16}H_{28}CuN_2O_{14}$ Bis-*O*-(β -D-xylopyranosyl)-L-serinatocopper(II)¹⁰²
 $C_{18}H_{20}BrNO_{10}S$ 6-*O*-(*p*-Bromophenylsulfonyl)gynocardin; (1*D*,4*D*,

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(95) C. E. Bugg and W. J. Cook, *Chem. Commun.*, 727-729 (1972).

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5L)-1-(β -D-glucopyranosyloxy)-4,5-dihydroxy-2-cyclopentene-1-carbonitrile¹⁰³

2. Nucleosides and Nucleotides

C₉H₁₂N₂O₆ Uridine¹⁰⁴

C₁₃H₁₅N₅O₃S·1/3H₂O 8,5'-Anhydro-2',3'-O-isopropylidene-8-mercaptopurine¹⁰⁵

C₁₄H₁₅ClN₄O₅ 6-Chloro-9-(3,4-di-O-acetyl-2-deoxy- β -D-erythro-pentopyranosyl)purine¹⁰⁶

NOTE ADDED IN PROOF

E and T Nomenclature for Furanose Rings

In the carbohydrate section, we have applied the descriptor that corresponds to the 18° sector of the pseudo-rotational phase-angle (P) diagram. For example, with 3T as P = 0° (as in Fig. 3 of Altona and Sundaralingam¹⁰⁷), all rings having P from -9° to +9° are described as *twist*, i.e., ${}^2T^3$, 3T , or 3T_2 , and all rings having P from +9° to +27° are described as *envelope*, i.e., 3E .

In regard to the nucleoside and nucleotide Section, this usage is in conflict with that employed in the existing literature, and therefore, to avoid confusion, we have limited the ideal *envelope* and *twist* descriptions to those rings for which the phase-angle is within 1° of the ideal value. For example, with 3T for P = -1° to +1°, the symbol 3E is used only for P +17° to +19°, and 3T_2 is applied for the intermediate values of P > +1° and <17°.

Clearly, a more definitive and uniform procedure would consist in reporting the value of the pseudo-rotational phase-angle, and we plan to do this in subsequent issues.

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ERRATA

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Page 375, formula 47. Move bottom two lines to right by one atom, and join the left-hand oxygen atom to the C of —C=N—R' .

Page 390, Table VII, column 1, entry 2 (under *Amino derivative*). For “O-(2-Diethylaminoethyl)cellulose” read “O-(2-Aminoethyl)cellulose.”

Page 392, Reference 652a. For “Vren” read “Uren”; move this name from column 2 to column 1 on page 430.

B 5
C 6
D 7
E 8
F 9
G 0
H 1
I 2
J 3